School of Molecular and Life Sciences

Development and Implementation of Next Generation Molecular Tool Kits for Environmental Applications

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material that has been accepted for the award of any other degree or diploma in any university.

Signature:

Date: 13 December 2019

Abstract

ENVIRONMENTAL DNA (eDNA) is used increasingly to map both past and present biodiversity, in terrestrial and marine environments. In recent years developments in high throughput sequencing technologies and metabarcoding methods have triggered a growth in DNA-based biodiversity surveys. Applied in the correct manner eDNA can generate useful data from a wide variety of complex substrates including; soil, sediment, bone fragments, scat, plankton and water. When this thesis was instigated there were very few metabarcoding studies focused on marine substrates. As such there was a need to: assess and enhance the existing molecular methods; research and develop new metabarcoding assays; and evaluate the use of eDNA in a marine setting. Of particular interest was the question of whether eDNA could respond to the challenge and become a method capable of true ecosystem-based monitoring across the tree-of-life.

Here, scat collected from the Australian sea lion (*Neophoca cinerea*), and an extensive collection of plankton samples provided by the Integrated Marine Observing System (IMOS) were utilised to optimise, design and test methodologies for application to marine eDNA samples. The two substrates provide a contrast in biodiversity assessment; while sea lions target larger fish, cephalopods and crustacea, plankton communities contain mostly microscopic taxa. At the outset of this thesis metabarcoding assays suitable for exploring marine eDNA were limited—this was particularly true for planktonic diversity. In response to this evident need, a range of new assays were designed and tested for efficacy.

Within the pages of this thesis, the depth of detail able to be extracted from eDNA is explored. The iconic Australian sea lion is revealed as a wideranging and opportunistic demersal Apex predator, which feeds on a wide range of mostly non-commercial marine fauna. Further, the samples of plankton provide us with a genetic glimpse of the complexity of the zooplankton populations from which they were taken.

The overall results, presented in three chapters, showcase the ability of eDNA and the 'tool kit' of assays to not only characterise dietary habits and describe biodiversity—but also to detect and map significant temporal and spatial trends in response to environmental stressors. More-over the results demonstrate how these genetic tools can provide the eDNA data necessary to combine with other abiotic variables and respond to the pressing need for marine environmental models that are able to predict the biotic reactions to the natural and anthropogenic biotic pressures facing our world's oceans.

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Preface—my motivation and invitation to readers

WHEN the title for this thesis was conceived, the ultimate direction of the research remained undefined. The motivation behind the project was always to 'develop and implement a next generation molecular tool kit' but there was uncertainty as to the nature of the 'environmental application' that the research would focus on.

Climate change and other anthropogenic stresses put marine habitats under increasing pressure and there are mounting concerns in regard to the health and viability of oceanic ecosystems. For these reasons, the research presented here was confined to marine applications and the use of environmentally non-destructive samples. It is recognised that oceanic ecosystems comprise countless complex interactions, yet the magnitude of the marine biodiversity within eukaryotes alone resulted in a further limitation of the research to the exploration of one kingdom, Animalia.

The combination of metabarcoding, 'high-throughput' or 'next-generation' deep sequencing (NGS) and environmental DNA (eDNA) has the ability to address a variety of environmental questions. These techniques have recently been used to analyse and monitor diet, biodiversity, the presence of cryptic and nocturnal species, and biosecurity (see Table 1.1). As genetic reference databases expand and methodologies are refined, the application of these techniques across diverse fields is rapidly growing (see Figure 1.1).

However, the ability to extract and sequence the constituents of eDNA from complex substrates is still relatively new and the methods used to extract and interrogate the DNA can require considerable optimisation and innovation, especially as the boundaries are pushed into unexplored territories.

The first chapter, Chapter one, provides an overview of why it is important to research and manage marine biodiversity, and examines historical and present day methodologies for achieving this. The overarching aims [of this thesis] and the approaches taken to achieve these aims are also described. You are welcome to engage further and read on!

My thanks to you all!

ANY work of consequence is not accomplished in isolation. Time alone will tell if the work enshrined within this thesis will have consequence, but in the meantime I must acknowledge and thank all the amazing people that have contributed their time and energy to help me realise this goal. I am sincerely grateful to you all.

Firstly and generally, an enormous thank you to my many supervisors and co-authors, I quite literally could not have achieved any of this without you, and to the ever growing members of the TrEnD Lab I thank you all for being there, coming to 'coffee club' and helping to keep me sane… I think!

Science may never come up with a better office communication system than the coffee break.

Earl Wilson

More particularly I wish to recognise and express my gratitude to the following individuals who have exceled in my estimation throughout my tenure. Firstly, Mike (Michael Bunce), you gave me the unique opportunity to embark on this journey. You then encouraged me to explore, define, refine, redefine, polish, refine again and finally (phew!) produce some top-notch quality science. Megan (Coghlan), you are my good friend and mentor, you engaged my interest, guided my endeavours and showed me science could be fun. You may have moved on to better things but TrEnD is missing your indefatigable spirit—and so am I. Michael (Stat), your skills with editing challenged me as a writer, and I emerged the better for it. You may be on the other side of the country, but you have not been forgotten. Simon (Jarman), I read so many of your papers before I found you were to be a part of our lab. Your research is inspirational and I have genuinely welcomed your interest and input into my work. Alicia ("Sensei" Grealy), you introduced me to Illustrator and the requirements for high quality graphics. Your keen eye, ability to spot an erroneous font from across the room, and your unsurpassed editing ability, all inspired me to achieve greater levels of excellence. Ant (Anthony Richardson) and Ben (Benjamin Saunders) you took on the

challenge and acquainted me with statistics using R and Primer. Your patience for my endless questions is a testament to your unique skills as educators. Matt (Mathew Power), you are the person to see whenever something needs doing or ordering. Your knowledge of workflows and the unseen processes, along with your unflappable manner, made you indispensible to my project. Dáithí (Murray), you introduced me to coding and enabled me to filter and process my data, and produce some robust results. I was sorry to see you go, but science's loss is medicine's gain. Katrina (West) and Frederik (Seersholm), my next-desk neighbours, and 'sounding boards', I am thankful you are always there for a clarification or for a quick question (or five!). Jason (Alexander) and Adam (Koziol), your extra-early coffee runs and lively conversations salvaged my sanity some mornings! There are so many others—Tiff, Nicole, Laura, Arne, Rose, Matt H, Klaus, Zoe, Miwa, Mieke, Kristen, Ali, Jacquie, David and Georgia—past and present and too numerous to mention here—but I thank you all!

It might take a village to raise a child but it takes a consortium to birth a thesis…

Last and, definitely, not least I thank my family. Jari (Mähönen), your belief in my abilities and your unending support (emotional and financial—including extending the mortgage on our house!) allowed me to follow this extended journey into marine biodiversity. You are a "keeper" and I am sure that when we married, you had no idea what you were in for—or how stressed I would become! Emily (Mähönen), I think I have been studying for almost all your remembered life. I am impressed by your ability to adapt and accommodate others, and step up to greater responsibilities when required. You are awesome and I am ever grateful that you are my daughter.

CHAPTER – ONE

Introduction and Literature Review

It is that range of biodiversity that we must care for - the whole thing - rather than just one or two stars.

David Attenborough

The sea, once it casts its spell, holds one in its net of wonder forever.

Jacques Yves Cousteau

1.1 Prelude

THIS chapter encompasses the historical and current framework for this research. Section 1.2 examines why marine environments are vital to protect. It also addresses the need to understand 'normality' in a marine ecosystem and why it is necessary to monitor marine environments.

The subsequent sections (1.3 and 1.4) address the two main and sometimes integrated historical approaches used to assess biodiversity, the observational and morphological methodologies. Section 1.5 briefly addresses the history of DNA sequencing and the emergence of reference databases. The developing use of environmental DNA (see Box 1.1 for definition) and metabarcoding is examined in section 1.6. This section commences with the provision of a discussion of the overall methods involved in analysing an environmental sample for metabarcoding and is followed by a discussion of the differing marine substrates used for eDNA extraction and analysis. A tabularised review of some of the marine eDNA literature (Table 1.1) is included within this section.

Box 1.1 A definition of environmental DNA

∞ Environmental DNA ∞

Environmental DNA (eDNA) is the DNA contained within a complex environmental mixture of DNA from a wide variety of organisms. The substrates containing eDNA are diverse and can include water, air, sediment, faeces and even bulk (community) samples (Taberlet et al., 2018). The DNA within an environmental sample can be intra- or extra- cellular, and can originate from living and dead organisms. The use of eDNA in research has expanded greatly in recent years (Figure 1.1) providing a testament to its utility.

Section 1.7 provides the close to this chapter; this section encompasses the scope and aims of the thesis. It also provides an outline of the structure of the remaining thesis and of the papers presented within each Chapter.

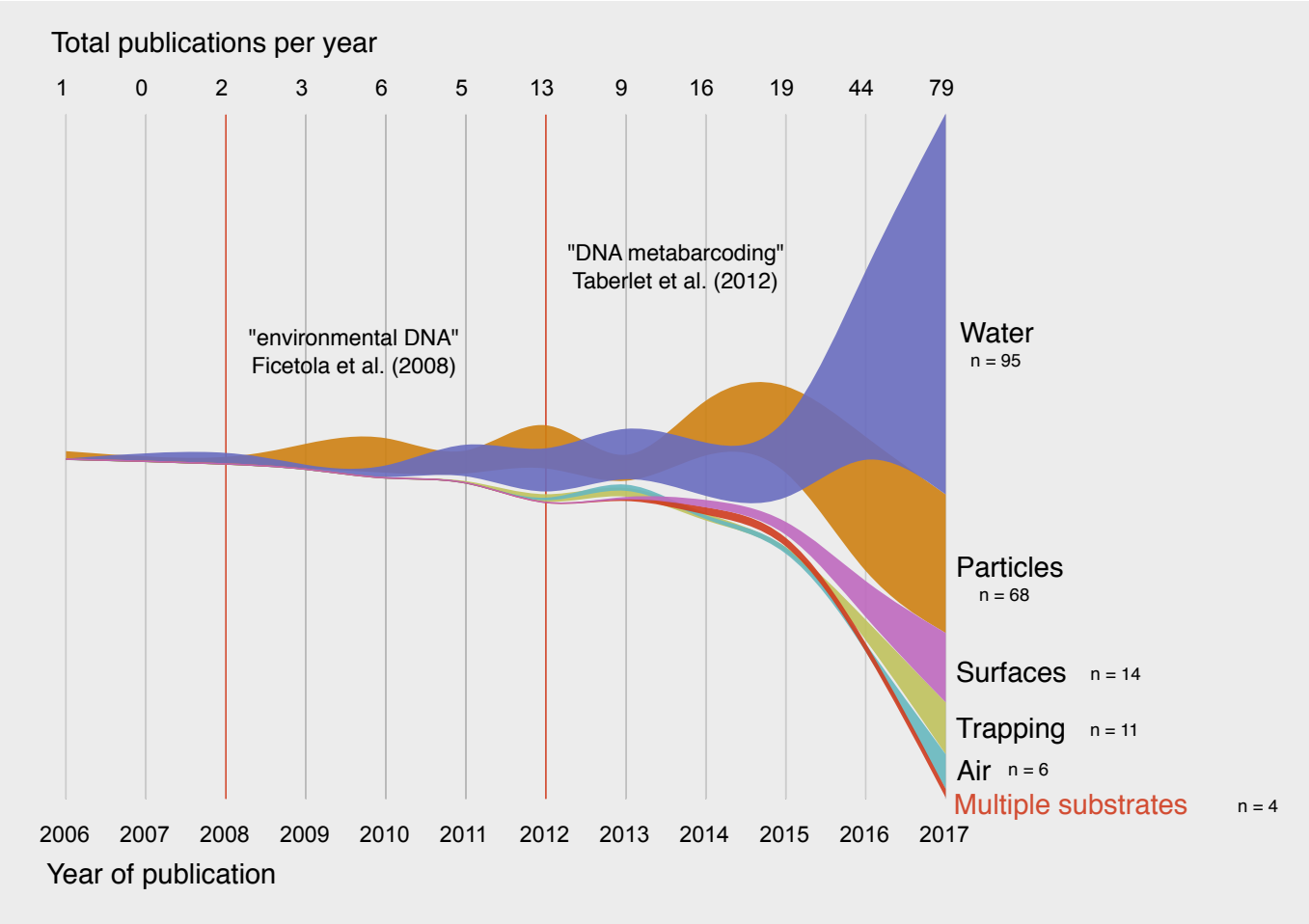

Figure 1.1: The rapid expansion of the number of studies using environmental DNA sequencing methodologies (Figure by Simon Jarman (2018): reproduced with permission)

1.2 Our waterlogged Earth—why study marine environments?

OCEANS conceal over two thirds of the Earth's surface forming about 90% of its inhabitable volume and providing the human population with 17% of its animal protein (FAO, 2016). The biomes they encompass are staggeringly diverse and the biodiversity of known and unknown marine eukaryotes alone are thought to be in excess of a million species, a majority of which remain uncharacterised (e. g. Mora et al., 2016).

The physical properties of the marine environment make the oceans inhospitable to humans, a fact which has, until relatively recently, protected its biota from excessive exploitation (see McCauley et al., 2015). While people have made use of marine resources since ancient times—there is evidence of pelagic fishing occurring as early as 42,000 years ago (O'Connor et al., 2011)—these forms of low-level subsistence harvesting had little impact on the overall diversity and abundance of marine life (see Lotze et al., 2006).

In contrast, marine defaunation has escalated in the last 150 to 300 years, particularly since the industrial revolution (see Lotze et al., 2006, McCauley et al., 2015 & Purcell, 2012). As human populations have increased the abundance of many larger marine species has decreased (see McCauley et al., 2015). In areas of denser human populations, marine species such as whales, mammals, reptiles, fish, birds and larger invertebrates have all been severely depleted, while levels of primary production, nutrient load, eutrophic plankton and sediment have all intensified (Lotze et al., 2006). Marine biomes are increasingly subject to pollution (e. g., microplastics; Lo and Chan, 2018), over exploitation, habitat disruption, introduction of invasive species and general oceanic industrialisation. Despite this, the numbers of marine faunal extinctions remain a fraction of those on land (see McCauley et al., 2015 & Leray and Knowlton, 2016); the current terrestrial extinction rate is thought to be at least 1000 times the background rate (see Pimm et al., 2014). This state is not completely due to the nature of the marine environment. Larger marine creatures are highly mobile and often widely dispersed and

thus can respond to changing conditions by changing or contracting their usual range (see McCauley et al., 2015). Additionally, it is entirely possible that some marine species have, and will, become extinct without ever being discovered (see Leray and Knowlton, 2016).

Adaptation to global climate change will be the greatest challenge facing almost every environment on earth as we continue through the 22nd century (IPCC, 2014). Mitigation of the degree to which climate change is allowed to progress and of its long-term effects are issues that face scientists now, and into the future. The effects of climate change are already documented (e. g., Hughes et al., 2018): increasing global temperatures are reducing polar ice caps with the result of rising sea levels. Additionally, there is evidence that, globally, marine heatwaves have increased in both frequency (Hughes et al., 2018) and duration over the last century (Oliver et al., 2018). Carbon dioxide is accumulating in the atmosphere and while the oceans are providing some relief from this by absorbing some of the excess (IPCC, 2014), the resulting acidification threatens the life cycles of many molluscs and other shell-reliant fauna (Gattuso et al., 2015, Poloczanska et al., 2013). There are already reports that increasing ocean temperatures are causing more temperate species to shift towards the poles, producing unknown implications for those species that usually reside there (Griffiths et al., 2017, Beaugrand et al., 2002).

All together, it is unknown how our marine ecosystems will respond to changes induced by climate change and climate variability (Richardson et al., 2012). However, a few studies provide rare insights: from 2001 to 2015, Wernberg et al. (2016) surveyed ecosystems across 2000 km^2 of a tropical to temperate transition zone within Western Australian waters. This area was of particular interest because its rate of warming was high (in the top 10%). Until 2010 kelp forests dominated more than 800 km of the coast, but by 2013 this coverage had been reduced by 43%. The researchers found that seaweed turfs had replaced the kelp in these areas and that the marine community had changed from species characteristic of temperate waters to those of sub-tropical or tropical waters. This change was attributed to the Western Australian marine heatwave

in 2011 and the nearly as warm sea surface temperatures experienced in 2012. More recently, Hughes et al. (2018) reports on some of the repercussions from the marine heatwave and extensive coral bleaching experienced by the Great Barrier Reef in 2016. The authors suggest that this event has already triggered a degradation of the reef and that this trend will continue until the climate stabilises. They propose that these changes will ultimately result in new, but more heat tolerant, reef assemblages. Matz et al. (2018) supports this finding, proffering a model that predicts that large-scale coral mortality will lead to coral extinctions, with environments later being reseeded by survivors and a migratory influx from other species.

These studies serve to illustrate the magnitude of the threat to our oceanic biomes. Significant habitat changes have far-reaching effects, affecting the behaviour and trophic interactions of those species that remain (O'Brien et al., 2018). The protection of marine environments—through the use of effective international agreements (Gattuso et al., 2015), strategic environmental schemes, harvest limits (Brown et al., 2011), moratoriums, and sanctuaries—is essential to if we wish to mitigate this trend. However, politically there are economic interests that must be balanced with ecological needs if strategies are to receive the support of governments and the broader community: in both the short and long term. Accordingly, baselines must be established (Borja et al., 2013), and suitable and persuasive scientific evidence must be provided to develop and support any proposed measures taken for the protection of climate, species, habitats and environments (Mokany et al., 2010). Sadly, the restoration of an ecosystem may require more than the mere removal of an anthropogenic stressor (Vacher et al., 2016), as the underlying functionality of an ecosystem is intrinsically linked to its biodiversity (Isbell et al., 2017).

The options for potential scientific investigation into the function of, and the anthropogenic impacts on, marine environments are wide-ranging, but from a marine biodiversity perspective they can be divided into three broad categories: observational, morphological and molecular, the first of which is addressed in the next section.

1.3 Uncovering macro marine biodiversity—observational studies

HISTORICALLY, a range of different approaches to research have been taken to assess and monitor both individual taxa and biodiversity. Observational studies can be used for behavioural, dietary and biodiversity research. Additionally, the methods used have the advantage of providing environmental context to any data collected. However, many marine observational studies are logistically difficult to implement. Specialist equipment, in the form of boats, submergible vehicles or cameras, diving apparatus and sampling gear or 'critter cams', are used to circumvent the hostile nature of the oceans and their large spatial scale. While such equipment allows species to be identified without collection, it is expensive and often requires specialist knowledge to use. Biodiversity studies via observation are also time and labour intensive, involve the use of skilled taxonomists and samples may need to be collected to make firm taxonomic identifications.

The collection of biodiversity data using observational methods is necessarily limited to those taxa that can be seen. The consequence being, that the large obvious diurnal animals, such as fish, larger corals, invertebrates and marine mammals, are more likely to be noticed and therefore over-represented as the proportion of biodiversity. Smaller, cryptic and shyer species are likely to be missed, as well as those that are nocturnal or crepuscular. Microscopic and larval fauna such as those found in zooplankton have little to no chance of morphological identification within an observational study. Leaving a large proportion of the base of the marine heterotrophic food chain unaccounted for. Consequently biodiversity data collected from observational studies is necessarily skewed. The following section addresses how morphological methods can better identify 'hidden' marine taxa

1.4 Uncovering unseen marine biodiversity—sample collection

TAXONOMY can also be used to identify microscopic taxa, and taxa concealed within substrates such as scat. For taxa to be morphologically identified in these cases, samples must be collected. In particular, the examination of scat or gastrointestinal samples from a generalist predator has been shown to provide valuable information about both the diet of the subject animal and the environment it occupies (Casper et al., 2007b). Morphological examination is also useful in the examination of plankton samples, where microscopy can aid in the identification a variety of minute taxa.

In the marine context, faecal samples from marine fauna can be difficult or impossible to collect; therefore, historically, researchers have turned to gastrointestinal samples. While gastrointestinal samples can be collected as the result of opportunistic or intentional necroscopies, collection from live animals can put the subject under tremendous stress and can, ultimately, result in the death of the animal. The ethical and logistic issues that this method raises have resulted in a call for less invasive methods (Casper et al., 2007a). Moreover, morphological examinations of scat or gastrointestinal samples depend on the rate of digestion of the prey and the taxonomic expertise of the researcher (a skill that is in decline). In larger marine carnivores, the survival of otoliths, beaks and exoskeletons from fish, cephalopods and crustacea (respectively) are necessary for the identification of prey (Casper et al., 2007b). Consequently species can be missed where identification is challenging or where digestion leaves no distinguishable residues, such as for soft-bodied organisms like jellies (Brown et al., 2012, McInnes et al., 2017).

Plankton samples, in contrast, are relatively easy to collect. However there are similar issues surrounding identification of the taxa. Both a skilled taxonomist and quality intact specimens, are required for reliable identifications. Eggs (Markle and Frost, 1985), larva, and cryptic species within a zooplankton sample are generally difficult to identify (Lindeque et al., 2013). Despite this, there is a wealth of valuable research and data that have been collected using morphology (e. g., Kelly et al., 2016, Kosobokova

and Hirche, 2016, Mackas et al., 2007, Mackas et al., 2012, Molinero et al., 2005, Rakhesh et al., 2006, Rice and Stewart, 2016, Schnack-Schiel et al., 2008, Schnack-Schiel et al., 2010, Williams, 1984)

Thus, the samples used for morphological methods can be difficult and/or expensive to collect and subsequent identifications are time consuming to perform. Increasingly, there is a need for ecological monitoring and research to produce rapid, cost effective and reliable results, especially where the outcome of the results can effect management decisions. The development of molecular techniques, and the ability to extract and sequence DNA from complex environmental substrates is beginning to fill this niche.

1.5 Sequencing of DNA—a brief history

DEOXYRIBONUCLEIC acid (DNA) consists of only four nucleotides (A: adenine, T: thymine, G: guanine and C: cytosine), yet together they code for a functional and structural roadmap for all life. The discovery of the structure of DNA was reported in 1953 (Watson and Crick), yet the ability to sequence (or read) DNA took many more years. In 1977 Sanger et al published a landmark paper describing a sequencing method using chain building deoxyribonucleotides (dNTPs) and radiolabelled chain-terminating dideoxynucleotides (ddNTPs) to produce identifiable replications (reads) of target sequences at all possible lengths for later viewing using gel electrophoresis. Sanger sequencing has been improved several times since then, including replacing the radiolabels with fluorescent tags and the development of capillary electrophoresis; thus, for many years it was the most commonly used sequencing technique (Heather & Chain, 2016). Although time consuming and expensive, Sanger sequencing remains relevant today.

The development of newer methods, tools and sequencing technologies, such as hot start polymerases, pyrosequencing (Roche 454), shotgun sequencing, semiconductor detection (Ion Torrent) and an increase in computer power and advanced analytical programs, has seen an explosion in sequence-based research. One consequence of this progression is the accumulation of reliable reference sequences from expertly identified specimens.

This build-up of data was acknowledged early on as a solution to an ongoing problem. Not all researchers with the need for taxonomic identification have the requisite expertise to identify a taxon; especially where the specimen is degraded, damaged, juvenile or cryptic. Samples can be taken from an unknown taxon for DNA sequencing and identification, but there was a need for reliable and accessible reference sequences.

The Barcode of Life Data System (BOLD) responded to this need; BOLD is a multinational consortium that aims to produce a barcode library for all extant

eukaryotes. This library accepts *Cytochrome c Oxidase subunit I (COI)* sequences, with a length of 500bp or more, from vouchered and identified specimens. It currently contains over 3 million sequences identified to a species level (Ratnasingham and Hebert, 2007). While this database is extremely useful and reliable, its utility is limited to *COI* sequences. Many researchers use sequencing assays that target an extensive array of taxa in order to pin down an identification. Unfortunately, the interspecies variability of *COI* makes it difficult to design *COI* sequencing assays (primers) that target a broad range of taxa.

The National Centre for Biotechnology Information has its own sequencing database, GenBank (Benson et al., 2014). This reference database incorporates the nucleotide sequences found on BOLD, but also accepts sequences from unvouchered specimens and sequences from a wide variety of alternate barcoding genes; such as *16S* and *18S RNA*. These two particular rRNA genes have areas of DNA which are highly conserved between related species, making them ideal for the design of more wide ranging sequencing assays.

The public databases are built on an enormous body of historical and ongoing molecular research involving the sequencing of individual taxa. Vouchered sequences typically originate from museum specimens, but there are cases where researchers have built there own reference database from species of interest and then deposited their sequences into a public database. These repositories not only deliver an avenue for scientists without taxonomic expertise to identify taxa from sequenced DNA, they also provide reference sequences to facilitate the design of custom assays to extricate sequences of interest.

In a marine eDNA context, the reference databases, and the research they are built on, have formed a solid foundation that allows extracted environmental DNA from marine samples to be sequenced and potentially identified.

1.6 Uncovering marine biodiversity—environmental DNA

ALL living cells contain DNA in one form or another, and this code can linger in the environment even after cell death as a part of environmental DNA. Environmental DNA (eDNA) is ubiquitous and while it may be relatively transient, it is continually being replenished with shed skin cells, saliva, mucous, waste, bacteria, microscopic eukaryotes, and other biota. There are various substrates that can yield useful amounts of eDNA, including sediment, faeces, water, and air (Taberlet et al., 2012). All contain a wealth of biodiversity information, but, depending on the substrate, the DNA present can be intracellular, extra cellular, abundant, at trace levels, and/or degraded (see Barnes and Turner, 2015 & Box 1.1). Until relatively recently there had been little prospect of taking advantage of such material. This situation changed with the introduction of next generation sequencing (Figure 1.1).

The sequencing and identification of eDNA requires the use of genetic 'barcodes'; small pieces of DNA with low intra-species variation and high inter-species variation that can be interrogated against a reference database (Joly et al., 2014) to produce a useful taxonomic identification, preferably with the ability to distinguish between species.

To identify the constituents of the environmental sample, the DNA must first be extracted from the substrate. The methods used for this vary depending upon the nature of the substrate. However all extractions require include steps to lyse cells, digest proteins and other cellular material and remove of salts, inhibitors and other contaminants, to obtain purified DNA.

Polymerase chain reaction (PCR) assays can then be used to enrich for the barcode necessary for the identification of taxa. Each assay consists of a pair of primers, small synthetic nucleotides (~20bp) that flank the 'barcode' of interest and facilitate replication (enrichment) of that 'barcode', such that its quantity is amplified relative to the non-target DNA.

Today, certain genes dominate reference genetic databases. Mitochondrial genes such as *Cytochrome c Oxidase subunit I (COI), 16S* and *18S ribosomal RNA (rRNA)* are all popular barcodes for Animalia, and the reference databases have many marine reference sequences (e. g., Benson et al., 2014, Ratnasingham and Hebert, 2007). Mitochondrial genes are typically targeted because they exist in much higher copy numbers than nuclear genes. This is particularly important when the target DNA is in low abundance.

Unfortunately, the assays used to produce the majority of database reference sequences cannot be used for eDNA as the 'barcode' inserts they generate (~600bp) are too long for both the degraded eDNA and the sequencing platforms used. Consequently there is a need for novel assays to be designed that produce smaller 'barcodes' (~100-200bp), suitable for degraded DNA, that target a wide range of marine taxa (Pompanon et al., 2012).

High-throughput sequencing allows for the use of metabarcoding (Taberlet et al., 2012) —a process that involves the use of assays that generate barcodes from a sizeable array of taxa within a mixture of DNA. High-throughput sequencing has the capacity to sequence—in parallel—millions of different sequences from a mixture and allows different samples to be combined into one sequencing 'run'. For each assay, each individual sample is assigned a pair of primers sporting unique genetic tags at the 5' end, which allows the samples to be pooled together and the generated sequences to be assigned back to the correct sample later. This permits a great many samples and assays to be sequenced together. Such a practice not only reduces sequencing costs, but also reduces the potential for cross-contamination between samples as the 'tags' are not reused.

The sequenced DNA metabarcodes can then be searched against an appropriate reference database. Depending on the project this can be a large public database, such as NCBI's GenBank (Benson et al., 2014) or a smaller and more focused custom database. This approach allows for the identification of the taxonomic origin of much of the DNA within a sample. While this identification is valuable, there is often a large

unassignable portion of DNA, especially within marine samples where the genetic references for barcodes are lacking. The option to analyse the sequence data in a taxonomy-free framework can be advantageous in these cases.

The formation of operational taxonomic units (OTUs) is a taxonomy-free way to examine genetic biodiversity. An OTU is a group of 'barcode' sequences that are—for example—97% similar to one another. These OTUs are then used to look for trends in the data without the need to identify the sequences. Figure 1.2 illustrates the eDNA workflow from environmental samples to data analysis.

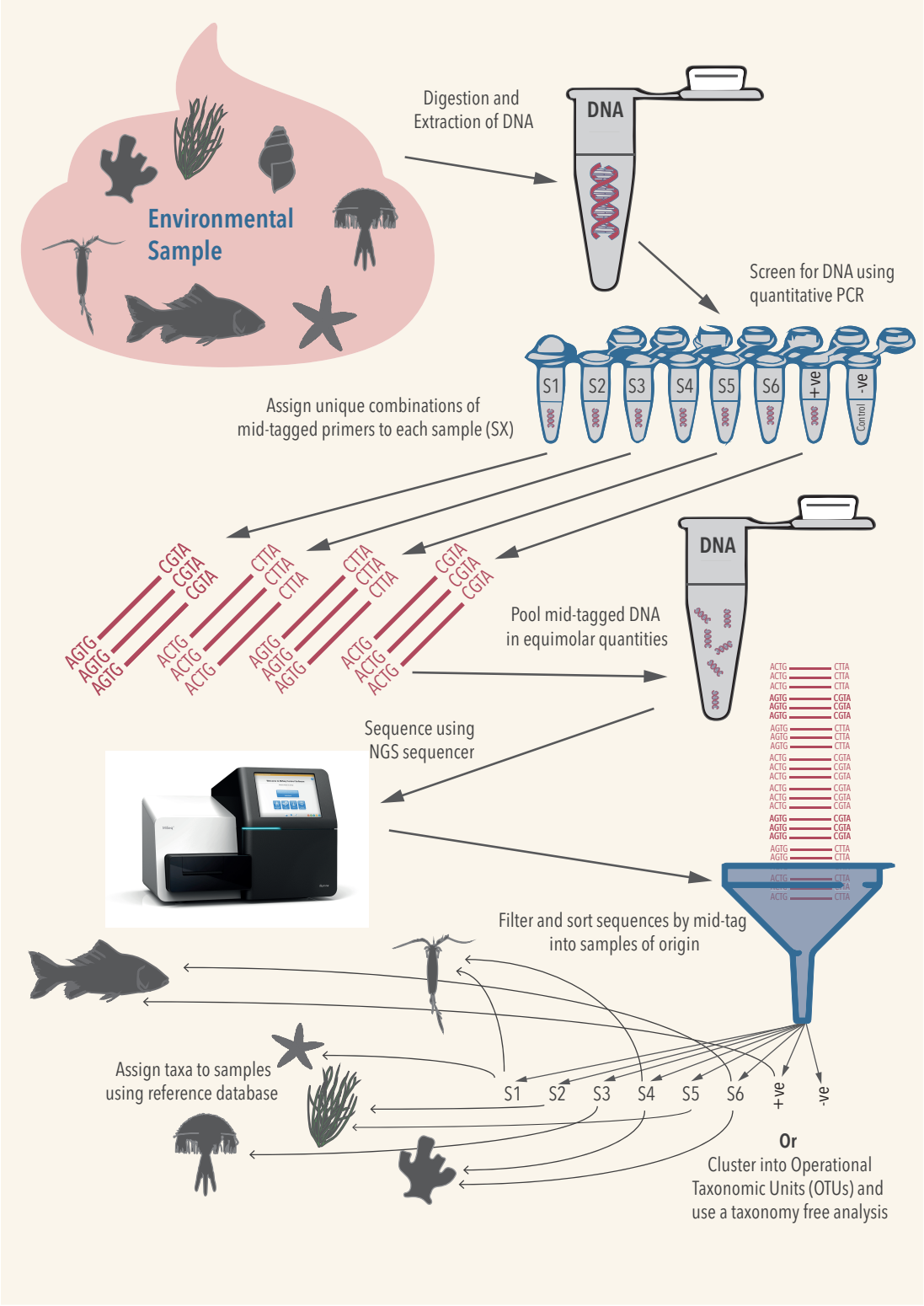

Figure 1.2: The environmental DNA workflow, from environmental samples to identification of environmental biodiversity.

The key advantage of using eDNA and metabarcoding is that the process is fast, accurate, non-invasive and relatively inexpensive. The methods can be used to identify hidden taxa, cryptic species (Thomsen and Willerslev, 2015), egg and larval forms (Osterhage et al., 2016), and for the construction of community data. It is particularly useful for detecting and monitoring endangered or invasive species (Bohmann et al., 2014).

The disadvantages of eDNA methodologies include: the inability to align species abundance with sequence quantity; PCR and assay bias, reference database inadequacies (Leray and Knowlton, 2016); and the incapacity to tell what stage of a life cycle the identified taxon is in (Clarke et al., 2017). Contamination is another potential problem when dealing with substrates containing trace amounts of DNA. Particular advantages and disadvantages will vary depending on the substrate selected.

Table 1.1 provides an insight into some of the recent marine eDNA sequencing research. This table demonstrates both the evolution of the sequencing methods over time, and the variety of substrates used, as well as the range of applications the methodologies have been put to. The succeeding sections give a brief overview of some of the marine substrates currently in use—and these are summarised in Table 1.2.

Table 1.1: Marine environmental DNA sequencing studies

Title	Genes	Sequencing method	Substrate	Targeted/Found	Range	Length of study	Reference
18S rRNA V9 metabarcoding for diet characterization: a critical evaluation with two sympatric zooplanktivorous fish species	*18S rRNA V9*	Illumina MiSeq	Gastrointestinal (84 samples)	Copepoda, Echinodermata, Protists	5 sites in Bay of Biscay, between France and Spain	May 2010	Albaina et al. (2016)
Viral to metazoan marine plankton nucleotide sequences from the Tara Oceans expedition	*16S* and *18S rDNA*	Illumina HiSeq	Plankton (1600 samples)	Viruses, prokaryotes, small eukaryotes, protists, metazoans	Worldwide	2009 – 2012	Alberti et al. (2017)
Biomonitoring of marine vertebrates in Monteray Bay using eDNA metabarcoding	*12S rDNA*	Illumina MiSeq	Seawater (12 samples)	Marine vertebrates – Chondrichthyes, Actinopterygii, Mammalia	Monteray Bay National Marine Sanctuary, California (10 sites)	29 Sept – 1 Oct 2015	Andruszkiewicz et al. (2017)
Molecular detection of marine nematodes from environmental samples: overcoming eukaryotic interference	*18S rDNA*	PCR cloning	Sediment (5 samples)	Nematodes	Saltash, Plymouth Sound, Plymouth Breakwater, Rame head & Humbar estuary, England		Bhadury et al. (2006)
Environmental DNA (eDNA) from the wake of whales: Droplet digital PCR for detection and species identification	Mitochondrial control region	PCR cloning to confirm digital droplet detection	Wake seawater (71 samples)	*Orcinus orca*	San Juan Islands in Canadian and US waters	Aug – Sept 2015	(Baker et al., 2018)
Comparison of morphological and DNA metabarcoding analyses of diets in exploited marine fishes	*COI, 16S* and *18S rDNA*	Roche 454	Gastrointestinal (151 samples)	Chondrichthyes, Actinopterygii, Nematodes, Platyhelminthes, Annelida, Crustacea	Bass Straight	Aug – Oct 2010 May 2011	Berry et al. (2015)
DNA metabarcoding for diet analysis and biodiversity: A case study using the endangered Australian sea lion (*Neophoca cinerea)*	*16S* and *12S rDNA* and *COI*	Illumina MiSeq & Roche 454	Scat (36 samples)	Actinopterygii, Mammalia, Aves, Crustacea, Cephalopoda	1,500km coast South-Western Western Australia (5 sites)	Oct 2012 – May 2013	Berry et al. (2017)
Assessing morphological and DNS-based diet analysis techniques in a generalist predator, the arrow squid *Nototodarus gouldi*	*16S rDNA*	PCR Cloning	Gastrointestinal (50 samples)	Actinopterygii, Mollusca, Crustacea	Great Australian Bight	Feb 2005 – Mar 2007	Braley et al. (2010)
Detecting prey from DNA in predator scats: A comparison with morphological analysis, using *Arctocephalus* seals fed a known diet	*28S rDNA* and *16S rDNA*	Gel comparison	Scat (182 samples)	*Arripis georgianus, Sillago robusta, S. flindersi, Upeneus tragula, Nototodarus sp., Trachurus novaezelandiae, Decapterus russelli, Sardinella lemuru, Amblygaster clupeoides, Scomber australasicus*, Penaeidae	Captive study with known diet	36 days	Casper et al. (2007a)

Title	Genes	Sequencing method	Substrate	Targeted/Found	Range	Length of study	Reference
Metabarcoding is powerful yet still blind: A comparative analysis of morphological and molecular surveys of seagrass communities	*COI* and *18S rDNA*	Roche 454	Sediment	Polychaeta, Malacostraca, Anthozoa, Bivalvia, Gastropoda, Sipinculidae	Brittany coast, western France (6 sites)	2 sites 2010 4 sites 2011	Cowart et al. (2015)
Pyrosequencing faecal DNA to determine diet of little penguins: is what goes in what comes out?	*16S rDNA*	Roche 454	Scat (100 wild samples)	Actinopterygii, Cephalopoda	Phillip and Rabbit Islands Victoria, Australia	Nov 22, 2007 – Jan 22, 2008	Deagle et al. (2010)
Genetic monitoring of open ocean biodiversity: An evaluation of DNA metabarcoding for processing continuous plankton recorder samples	*COI*	Illumina MiSeq	Plankton (79 samples)	Predominantly metazoans	The Southern Ocean between Tasmania and Macquarie Island	Overnight	Deagle et al. (2017)
Studying seabird diet through genetic analysis of Faeces: A case study on Macaroni penguins (*Eudyptes chrysolophus*)	*16S* and *28S rDNA*	PCR cloning	Scat (88 samples)	Euphausiids, Actinopterygii, Amphipoda, Cephalopoda, Chaetognatha	Heard Island	Dec 20, 2003 & Feb 16, 2004	Deagle et al. (2007)
Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces	*16S, 18S* and *28S rDNA*	Roche 454 & PCR cloning	Scat (270 samples)	Chondrichthyes, Actinopterygii, Cephalopoda	South Eastern Australia between Victoria and Tasmania (3 Colonies)	Dec 27 2007 – Jan 24 2008	Deagle et al. (2009)
Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions	*16S* and *28S rDNA*	PCR cloning and gel comparison	Scat (40 samples)	Pollock, Salmon, Capelin, Smelt, Herring, Squid	Captive study	Jul – Sept 2003	Deagle et al. (2005)
Colony location and captivity influence the gut microbial community composition of the Australian sea lion (*Neophoca cinerea*)	*16S rDNA*	Illumina MiSeq	Scat (43 samples)	Firmicutes, Proteobacteria, Fusobacteria Chloroflexi, Cyanobacteria	South-Western Australia and captive population	Wild; Mar 2009 – Sept 2010, Captive; Mar 2011 – May 2013	Delport et al. (2016)
Identifying prey items from New Zealand fur seal (*Arctocephalus forsteri*) faeces using massive parallel sequencing	*16S, 28S* and *18S rDNA*	Illumina MiSeq	Scat (110 samples)	Chondrichthyes, Actinopterygii, Cephalopoda	Full geographic range of New Zealand (5 sites)	2012 – 2014	Emami-Khoyi et al. (2016)
Second-generation environmental sequencing unmasks marine metazoan biodiversity	*nSSU rDNA*	Roche 454	Sediment (24 samples)	Nematoda, Platyhelminthes, Mollusca, Annelida, Arthropoda, Stramenopiles, Rhizaria, Echinodermata	Prestwick and Littlehampton, United Kingdom	Summer 2007	Fonseca et al. (2010)
Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine animals	*12S rDNA*	PCR cloning	Seawater (50 harbour; 24 Baltic sea)	Harbour porpoise (*Phocoena phocoena*)	Kerteminde, Denmark and western Baltic sea	August 2011	Foote et al. (2012)

Title	Genes	Sequencing method	Substrate	Targeted/Found	Range	Length of study	Reference
Estimation of a Killer Whale (*Orcinus orca*) population's diet using sequencing analysis of DNA from feces	*16S rDNA*	Illumina MiSeq	Scat (175 samples)	Actinopterygii, *Orcinus orca*	San Juan Islands in Canadian and US waters	May – Sept of 2006 – 2011	Ford et al. (2016)
The Tara Oceans voyage reveals global diversity and distribution patterns of marine planktonic ciliates	*18S rDNA*	Illumina HiSeq	Plankton (1600 samples)	Ciliophora	Worldwide	2009 – 2012	Gimmler et al. (2016)
Fish consumption of harbour seals (*Phoca vitulina*) in north western Iceland assessed by DNA metabarcoding and morphological analysis	*16S rDNA*	Roche 454	Scat (116 samples)	Actinopterygii, Aves	Vatnsnes Peninsula, Iceland	May – Aug 2010 & 2011	Granquist et al. (2018)
Deep-sea, deep-sequencing: Metabarcoding extracellular DNA from sediments of marine canyons	*18S rDNA*	Illumina MiSeq	Sediment (20 samples)	Metazoa, Alveolata, Stramenophiles, Rhizaria	Western Mediterranean, Spain (4 marine canyon sites)	March and June 2012	Guardiola et al. (2015)
Assessing the trophic ecology of top predators across a recolonisation frontier using DNA metabarcoding of diets	*16S rDNA*	Illumina MiSeq	Scat (112 samples)	Actinopterygii, Cephalopoda, Malacostraca, Aves	Montague Island and Jervis Bay, NSW	Jan – Apr & Sept 2014	Hardy et al. (2017)
Comparison of morphological and next generation DNA sequencing methods for assessing zooplankton assemblages	*COI* and *28S rDNA*	Roche 454	Zooplankton	Calanoida, Euphausiacea, Decapoda, Polychaeta, Pleuronectiformes	Northern Monteray Bay, California (4 main sites)	Aug 12 – 16, 2013	Harvey et al. (2017)
A metagenetic approach for revealing community structure of marine planktonic copepods	*LSU rDNA*	Roche 454	Zooplankton (3 field samples: 1 artificial sample)	Calanoida, Cyclopoida, Poecilostomatoida, Harpacticoida	Kuroshio current, Japan (3 sites)	May 9 – 18, 2011	Hirai et al. (2015)
Metagenetic community analysis of epipelagic planktonic copepods in the tropical and subtropical Pacific	*LSU rDNA*	Roche 454	Zooplankton (20 samples)	Copepods	4 regions in the tropical and sub-tropical Pacific (16 sites)	May 2011 – Feb 2012	Hirai and Tsuda (2015)
Adélie penguin population diet monitoring by analysis of food DNA in scats	*SSU rDNA*	Ion Torrent	Scat (389 samples)	Actinopterygii, Crustacea, Porifera, Cnidaria, Cephalopoda, Algae etc	Australian Antarctic Territory	2008 – 2012	Jarman et al. (2013)
Using environmental DNA to census marine fishes in a large mesocosm	*12S rRNA*	Illumina MiSeq	Seawater (2 samples)	Actinopterygii, Chondrichthyes, *Chelonia mydas*	Monteray Bay Aquarium	Feb 2013	Kelly et al. (2014)

Title	Genes	Sequencing method	Substrate	Targeted/Found	Range	Length of study	Reference
Ultra-deep sequencing of foraminiferal microbarcodes unveils hidden richness of early monothalamous lineages in deep-sea sediments	*37f-SSU rDNA*	Illumina Solexa GAII	Sediment (31 samples)	Foraminifera	Arctic Ocean, Baffin Bay, Caribbean Sea, Pacific Ocean, Weddell Sea	2007 – 2008	Lecroq et al. (2011)
Next generation sequencing reveals the hidden diversity of zooplankton assemblages	*18S rRNA*	Roche 454	Zooplankton (4 samples)	Annelida, Arthropoda, Chordata, Chaetognatha, Cnidaria, Chromista, Ctenophora, Echinodermata, Mollusca, Nematoda, Platyhelminthes	Western Channel Observatory (English Channel)	Sept 2010 & Jan 2011	Lindeque et al. (2013)
High occurrence of jellyfish predation by Black-browed and Campbell albatross identified by DNA metabarcoding	*18S rDNA*	Illumina MiSeq	Scat (1460 samples)	Actinopterygii, Scyphozoa, Crustacea, Cephalopoda, Hydrozoa, Chondrochthyes, Anthozoa, Ctenophora, Tunicata	Island sites from 40°S to 60°S around Antarctica	Various sites 2013 – 2016	McInnes et al. (2017)
Simultaneous DNA-based diet analysis of breeding, non-breeding and chick Adélie penguins	*SSU rRNA*	Ion Torrent	Scat (348 samples)	Scyphozoa, actinopterygii, euphausiids, amphipods, calanoida	Béchervaise Island and Whitney Point, East Antarctica	23 – 28 Dec 2012, 4 – 7 Jan & 23 – 26 Jan 2013	McInnes et al. (2016)
MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species	*12S rDNA*	Illumina MiSeq	Seawater (18 samples)	Actinopterygii, Chondrichthyes	Okinawa Churaumi Aquarium, Japan – 4 tanks with variable conditions and a coral reef nearby	June 2014	Miya et al. (2015)
Planktonic foraminifera-derived environmental DNA extracted from abyssal sediments preserves patterns of plankton macroecology	*37f-SSU rDNA*	Illumina Solexa GAII	Sediment (31 samples)	Foraminifera	Caribbean sea to Arctic Ocean	2007 – 2008	Morard et al. (2017)
DNA-based faecal dietary analysis: A comparison of qPCR and high-throughput sequencing approaches	*16S rDNA*	PCR cloning & Roche 454	Scat (47 & 52 samples)	Actinopterygii	Penguin Island, Western Australia	Aug 2008 – Sep 2009 & Oct – Dec 2010	Murray et al. (2011)
Determining the diet of larvae of the red rock lobster (*Jasus edwardsii*) using high-throughput DNA sequencing techniques	*18S rDNA*	Roche 454	mid gut gland (19 samples)	Arthropoda, Chordata, Cnidaria, Ctenophora, Echinodermata, Mollusca, Radiolaria, Ascomycota, Basidiomycota, Chromalveloata, Viridiplantae	Waters off the eastern coast of New Zealand	Oct 2009, May 2010, Feb & May 2011	O'Rorke et al. (2013)
Environmental monitoring through protest next-generation sequencing metabarcoding: assessing the impact of fish farming on benthic foraminifera communities	*37f-SSU rDNA*	Illumina MiSeq	Sediment (45 samples)	Foraminifera	Salmon farms at Loch Linnhe and Loch Creran, Scotland (3 sites)		Pawlowski et al. (2014)

Title	Genes	Sequencing method	Substrate	Targeted/Found	Range	Length of study	Reference
Fine-scale diet of the Australian sea-lion (*Neophoca cinerea*) using DNA based analysis of faeces	*16S rDNA*	PCR cloning	Scat (12 samples)	Actinopterygii, Cephalopoda	Lilliput Island and Kangaroo Island, South Australia	Jul – Aug 2006	Peters et al. (2014)
PCR-based techniques to determine diet of the Australian sea lion (*Neophoca cinerea*): a comparison with morphological analysis	*COI* and *16S rDNA*	PCR cloning	Scat (58 samples)	*Sepioteuthis australis, Mustelus antarcticus, Arripis georgianus, Pelates octolineatus*	Captive study	Jan – Feb 2006	Peters et al. (2015)
Evaluating detection limits of next-generation sequencing for the surveillance and monitoring of international marine pests	*COI* and *18S rDNA*	PCR cloning & Roche 454	Plankton (1 sample) and sediment (1 sample)	*Asterias amurensis, Carcinus maenas, Ciona intestinalis & savignyi, Corbula amurensis & gibba, Perna canaliculu & perna, Sabella spalanzanii*	Tasman Bay, New Zealand	May 1, 2012 & Aug 8, 2012	Pochon et al. (2013)
Targeted search for actinomycetes from nearshore and deep-sea marine sediments	*16S rDNA*	PCR cloning	Sediment (5 samples)	Actinomycetales	Canary Basin and South Pacific Gyre		Prieto-Davó et al. (2013)
Analysis of diversity of chromophytic phytoplankton in a mangrove ecosystem using *rbcL* gene sequencing	*rbcL*	PCR cloning	Seawater (10 samples)	Phytoplankton	Mooriganga estuary in the Indian Sundarbans	Apr and Dec 2010 & Mar 2011	Samanta and Bhadury (2014)
Population characteristics of a large whale shark aggregation inferred from seawater environmental DNA	Mitochondrial Control region	Illumina MiSeq	Seawater (20 samples)	*Rhincodon typus*	Al Shaheen oil field Qatar (15 sites)	May 2013, May – Jun 2015	Sigsgaard et al. (2016)
Seawater environmental DNA reflects seasonality of a coastal fish community	*12S rDNA*	Illumina MiSeq	Seawater (26 samples)	Actinopterygii	Skovshoved Harbour, Zealand, Denmark	Aug 2013 – Aug 2014	Sigsgaard et al. (2017)
Real-time PCR detection of *Didemnum perlucidum* (Monniot, 1983) and *Didemnum vexillim* (Knott, 2002) in an applied routine marine biosecurity context	*COI*	Real time PCR	Seawater (25 samples)	*Didemnum perlucidum, D. vexillum*	Hillarys Boat Harbour and Swan River Estuary Western Australia (9 sites)	May, Aug & Dec 2014 Jan 2015	Simpson et al. (2016)
Ecosystem biomonitoring with eDNA: metabarcoding across the tree of life in a tropical marine environment	Shotgun and *COI, 18S, 16S* and *23S rDNA*	Illumina NextSeq and MiSeq	Seawater (9 samples)	Total diversity	Coral Bay, western Australia (single site)	Mar 17 – 18, 2015	Stat et al. (2017)
Aquatic environmental DNA detects seasonal fish abundance and habitat preference in an urban estuary	*12S rDNA*	Illumina MiSeq	Seawater (76 samples)	Actinopterygii	Lower Hudson River estuary, New York (2 sites)	Jan – Jul 2016	Stoeckle et al. (2017)

Title	Genes	Sequencing method	Substrate	Targeted/Found	Range	Length of study	Reference
Dietary analysis of marine fish species: Enhancing the detection of prey-specific DNA sequences via high-throughput sequencing using blocking primers	*COI*	Illumina MiSeq	Gastrointestinal (222 samples)	Actinopterygii, Malacostraca, Hexanauplia, Cnidaria, Mollusca, Aves	Donghai Island, China	Mar, Jun, Sept, & Dec 2013	Su et al. (2017)
Improving accuracy of DNA diet estimates using food tissue control materials and an evaluation of proxies for digestion bias	*16S rDNA*	Ion Torrent PGM	Scat (48 samples)	*Mallotus villosus, Clupea pallasii, Scomber japonicas, Loligo opalescens*	Captive study		Thomas et al. (2014)
Detection of a diverse marine fish fauna using environmental DNA from seawater samples	*Cytochrome b*	Roche 454	Seawater (3 samples)	Actinopterygii, Aves	Denmark	2011	Thomsen et al. (2012)
Blocking primers to enhance PCR amplification of rare sequences in mixed samples – a case study on prey DNA in Antarctic krill stomachs	*28S rDNA*	PCR cloning	Gastrointestinal	Algae, Ciliates, Krill	Eastern Antarctic waters	Mar & Sept – Oct 2007	Vestheim and Jarman (2008)
The impact of artificial surfaces on marine bacterial and eukaryotic biofouling assemblages: A high-throughput sequencing analysis	*16S* and *18S rDNA*	Illumina MiSeq	Settlement plates (60 samples)	Bacteria and Eukaryotes	Auckland Westhaven Marina (New Zealand)	23 June – 20 October 2015	Von Ammon et al. (2018)
High sensitivity of 454 pyrosequencing for detection of rare species in aquatic communities	*V4-nSSU*	Roche 454	Plankton spiked with known molluscs and crustaceans	*Argopecten irradians, apostichopus japonicas, Limnoperna fortune, Asellus aquaticus*	Atlantic coast of North America (Bayside and Hawksbury)		Zhan et al. (2013)

1.6.1 Marine scat

Dietary analysis is central to the discipline of ecology (see O'Rorke et al., 2012, Deagle et al., 2010 & Ford et al., 2016), yet until comparatively recently the practice relied on either observational studies, or morphological examination of gastrointestinal or faecal samples. Scat from a generalist predator can be used as a proxy for biodiversity, as they sample from a diverse range of taxa (Boyer et al., 2015, Jarman et al., 2013, McInnes et al., 2017). Furthermore larger marine mammals can exert a substantial influence on the biodiversity of their habitat (Casper et al., 2007b).

Despite the advantage of obtaining dietary data along with biodiversity information, there are still issues with the manner of sample collection, and the biodiversity identified is limited by what the predator eats. Another issue is that of an overabundance of host and bacterial DNA within the sample. To circumvent this issue, assays must be carefully designed to exclude these inherent non-target DNA contaminants. Where this is not possible a blocking primer can be used to exclude host DNA (see Vestheim and Jarman, 2008). This is a small synthetic oligonucleotide that is specific to the host's 'barcode', it prevents enrichment by binding to the host's 'barcode' and inhibiting amplification. However, it is often necessary, especially where the data is being used for dietary analysis, to confirm the origin of the scat. This is where a species-specific assay may be used.

1.6.2 Seawater

The use of seawater in eDNA biodiversity studies was novel at the outset of this thesis but is now heavily used (see Figure 1.1). With comparatively little equipment, water can be easily sampled, and filtered for cells and exogenous DNA, at or near the sampling site. The resulting filter paper containing the sample is then placed in a small sample bag and placed on ice until it can be frozen prior to extraction. The small size of the filters allows the researcher store DNA from many samples within a confined space.

All marine animals shed cells and DNA into their surroundings. The amount shed is dependent on the species, but the DNA will remain within the animal's habitat until degraded or dispersed. The result being that the DNA collected will only reflect taxa that inhabit or have relatively recently traversed the area. It is predominantly this DNA that is sampled when the water is filtered.

The DNA collected from within this substrate is likely to be highly degraded and of poorer quality. Therefore PCR assays targeting short barcode regions need to be used to maximise the amount of information taken from the sample. The choice of assay will also determine what taxa will be targeted. For example if the water sample was taken from a coral reef then an assay targeting corals would be prudent, but there are likely also to be fish, cnidarians, molluscs or other taxa present. One solution, which was used in a co-authored paper (Stat et al., 2017— see Appendix), is to employ a multiple assay approach to target a larger range of taxa.

1.6.3 Marine sediment

Many marine taxa live on or within the sediment beneath the ocean. Additionally faecal material and the remains of animals that have died will tend to accumulate here to be consumed, decomposed, or preserved. The substrate reflects much of the biodiversity of the ecosystem and can provide a wealth of information (Morard et al., 2017). One study (Turner et al., 2015) in a freshwater environment showed that a gram of sediment contained Carp DNA (*Hypophthalmichthys* spp.) that was 8-1800 times more concentrated than within a millilitre of water. However, the sampling of sediment is comparatively problematic and the difficulties increase with the depth of the water above the sample site.

Sediment derived eDNA when compared with plankton is not as influenced by habitat depth, seasonal or other cyclic changes and provides an archive of biodiversity (Morard et al., 2017). However, ancient eDNA can also persist in sediment, which can

potentially confound analyses centred on present day biodiversity if care is not taken to understand the stratification of samples (Thomsen and Willerslev, 2015).

1.6.4 Settlement Plates

Settlement plates are a type of sampling tool that can be placed at sites of interest to assess biofouling (von Ammon et al. 2018) or the accumulated biodiversity over time. While the plates collect debris settled out from the water above, they also can be colonised by molluscs and other animals looking for a solid surface to inhabit. Depending on age, removal of a sample from a settlement plates can be difficult. Samples may be removed using sterile sponges (von Ammon et al. 2018) but often must be scraped off and homogenised to allow for subsampling. The presence of molluscs and other hard taxa can hamper this process. This type of sampling has been adopted for use in biosecurity (Pochon et al., 2013) as it allows for insight into biomes that form on marine surfaces such as the hulls of ships.

1.6.5 Plankton

Plankton is made up of both plants (autotrophic phytoplankton) and animals (heterotrophic zooplankton). It forms the basis of much of the marine food web and its biodiversity is enormous (Richardson, 2009). The collection of plankton generally occurs using continuous plankton recorders, Niskin bottles, drop nets or plankton tows (Davies et al., 2016). The process is relatively easy and can be performed alongside other research activities. The resulting samples are relatively small and, for molecular purposes, can be stored on ice until frozen.

Many plankton species are not present in the current genetic reference databases. Even with broad coverage assays much of the taxa detected remains unidentified. Plankton populations also respond rapidly to changing environments and so to get a clear picture

of biodiversity, multiple samples at different time points need to be made from a single sites.

1.6.6 Substrate selection

Taxonomic niche theory states that different taxa inhabit, or are found in, different substrates; consequently, where there is a choice, the selection of an appropriate substrate will depend upon the taxa the researcher is most interested in. The results obtained from any sample are a product of the DNA present in the sample and the efficacy of the assay selected to detect it (Pompanon et al., 2012). Accordingly some knowledge of what taxa to expect is needed to target the most appropriate assays (see Braley et al., 2010).

Table 1.2 contains a summary of some of the advantages and disadvantages of the marine eDNA substrates addressed in the previous sections of this chapter. The following and final section looks at the scope and aims of this thesis as well as its structure.

Table 1.2: Summary of some of the advantages and disadvantages of several eDNA marine substrates

Marine substrate	Advantages	Disadvantages
Plankton	Small sample size Ease of collection and storage Sampling can occur alongside other research activities Broad range of biodiversity Identification of taxa unable to be identified morphologically Identification of cryptic and larval taxa Can contain DNA from larger taxa that predate plankton	Broad range of biodiversity Requires several assays Many taxa are not found on genetic reference databases
Scat	A non-harmful method to obtain dietary knowledge Can be used as a proxy for biodiversity Can provide valuable genetic information about the host	Abounds with host and bacterial DNA Data limited to dietary preferences of host Can be difficult to collect where the host defecates underwater Prior knowledge of some dietary habits required to target assays
Seawater	Ease of collection, processing and storage of samples Sampling can occur alongside other research activities Provides data for cryptic and hidden species Provides current biodiversity information Can be used to monitor invasive species	Abounds with microbial taxa Highly mobile taxa may be missed DNA can be rapidly dispersed in areas with greater water movement DNA can be low quantity and is often degraded
Sediment	Contains DNA from both living and dead taxa Not influenced by habitat depth, seasonal or other changes Can form strata and provide an archive of historical biodiversity	Temporal data can be misleading if sediment strata is not maintained or understood Difficulty in sampling increases with the water depth
Settlement plates	Can be placed in areas of interest Useful for assessing taxa that colonise hard surfaces Can be used for biosecurity and to investigate biofouling	Samples can be difficult to remove for analysis Samples require time to accumulate

1.7 Close—thesis housekeeping

THE aims and scope of this thesis are quite general. The first aim was to develop and optimise molecular techniques to sample, extract and analyse eDNA from marine substrates. When research for this thesis was commenced in 2014 the methodologies used to investigate eDNA were still quite novel (Figure 1.1). Environmental DNA studies of marine environments were rare, and those incorporating metabarcoding techniques were even rarer (Table 1.1). Thus, there were few systems and assays available for the metabarcoding of marine samples. This thesis reports on development of new techniques to extract and sequence eDNA from marine scat and plankton; however during the tenure of this thesis methods for seawater were also optimised. These techniques are reported in a co-authored paper (Stat et al., 2017—see Appendix).

The second aim was to evaluate the methodologies developed in terms of their ability to provide an understanding of the biodiversity contained within the substrate. The developed methodologies needed to provide a substantial insight into the taxa contained within the substrate. For this reason a multi-gene metabarcoding approach was adopted, this method maximises the response from the genetic material present in the substrate.

The final aim was to apply the extracted information and address topical marine environmental concerns. The data obtained needed to be useful, and so the substrates and assays chosen for the research had the capability of answering some key environmental questions.

The three aims formed the foundation for each study described within this thesis and throughout each study these aims are targeted in a different way. In Chapter Two, eDNA extracted from the scat of the endangered Australian sea lion (*Neophoca cinerea*) was used to develop three new assays that provided a rare insight into both the sea lion's diet and the biodiversity of its habitat. This data showed that although the sea lion was targeting some commercial species, their dietary choices were more opportunistic. In

Chapters Three and Four, plankton samples were used to refine extraction techniques, develop more than twenty new assays, assemble a metabarcoding marine 'tool kit' and finally demonstrate the ability of the resulting eDNA data to reflect both spatial and temporal stressors such as regular and irregular seasonal changes. Thus providing a new avenue to monitor and understand the very base of the marine food web.

1.6.1 Thesis structure

This thesis is presented as a hybrid thesis for publication. Chapters Two through Four provide accounts of particular research topics in an as-published, submitted or ready-for-publication format. Each of these chapters is prefaced with an explanation of the relevance of the topic to the overall thesis and includes an acknowledgement of all contributions by co-authors and author declarations. The chapters are then closed, by assessing the significance of the research, providing information as to advances subsequent to the research and indicating how the current chapter relates to the next.

Chapter Five includes a general discussion about the way in which the research has met the aims of the thesis and examines what could be done to advance further the research in these areas. This final chapter is followed by the Appendix, which contains a facsimile of the published chapter and the relevant co-author permissions for all the chapters. It also contains relevant published co-author papers that have resulted from research undertaken during the tenure of this thesis.

The following chapter outlines a study using faeces from an endangered endemic pinniped. While this animal had been the focus of several observational and morphological studies, this research was the first to use next generation sequencing and multi-gene metabarcoding to gain a significant insight into the animal's diet and the biodiversity of its habitat.

1.7 References

ALBAINA, A., AGUIRRE, M., ABAD, D., SANTOS, M. & ESTONBA, A. 2016. 18S rRNA V9 metabarcoding for diet characterization: a critical evaluation with two sympatric zooplanktivorous fish species. *Ecology and evolution,* 6**,** 1809-1824.

ALBERTI, A., POULAIN, J., ENGELEN, S., LABADIE, K., ROMAC, S., FERRERA, I., ALBINI, G., AURY, J.-M., BELSER, C. & BERTRAND, A. 2017. Viral to metazoan marine plankton nucleotide sequences from the Tara Oceans expedition. *Scientific data,* 4**,** sdata201793.

ANDRUSZKIEWICZ, E. A., STARKS, H. A., CHAVEZ, F. P., SASSOUBRE, L. M., BLOCK, B. A. & BOEHM, A. B. 2017. Biomonitoring of marine vertebrates in Monterey Bay using eDNA metabarcoding. *PLOS ONE,* 12**,** e0176343.

BAKER, C. S., STEEL, D., NIEUKIRK, S. & KLINCK, H. 2018. Environmental DNA (eDNA) From the Wake of the Whales: Droplet Digital PCR for Detection and Species Identification. *Frontiers in Marine Science,* 5.

BARNES, M. A. & TURNER, C. R. 2015. The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics,* 17**,** 1-17.

BEAUGRAND, G., REID, P. C., IBANEZ, F., LINDLEY, J. A. & EDWARDS, M. 2002. Reorganization of North Atlantic marine copepod biodiversity and climate. *Science,* 296**,** 1692-4.

BENSON, D. A., CLARK, K., KARSCH-MIZRACHI, I., LIPMAN, D. J., OSTELL, J. & SAYERS, E. W. 2014. GenBank. *Nucleic Acids Research,* 42**,** D32-D37.

BERRY, O., BULMAN, C., BUNCE, M., COGHLAN, M., MURRAY, D. C. & WARD, R. D. 2015. Comparison of morphological and DNA metabarcoding analyses of diets in exploited marine fishes. *Marine Ecology Progress Series,* 540**,** 167-181.

BERRY, T. E., OSTERRIEDER, S. K., MURRAY, D. C., COGHLAN, M. L., RICHARDSON, A. J., GREALY, A. K., STAT, M., BEJDER, L. & BUNCE, M. 2017. DNA metabarcoding for diet analysis and biodiversity: A case study using the endangered Australian sea lion (*Neophoca cinerea*). *Ecol Evol,* 7**,** 5435-5453.

BERRY, T. E., SAUNDERS, B. J., COGHLAN, M. L., STAT, M., JARMAN, S., RICHARDSON, A. J., DAVIES, C. H., BERRY, O., HARVEY, E. S. & BUNCE, M. 2019. Marine environmental DNA biomonitoring reveals seasonal patterns in biodiversity and identifies ecosystem responses to anomalous climatic events. *PLOS Genetics,* 5(2). e1007943

BHADURY, P., AUSTEN, M. C., BILTON, D. P., LAMBSHEAD, P.J. D., ROGERS, A. D. & SMERDON, G. R. 2006. Molecular detection of marine nematodes from environmental samples: overcoming eukaryotic interference. *Aquatic Microbial Ecology,* 44**,** 97-103.

BOHMANN, K., EVANS, A., GILBERT, M. T., CARVALHO, G. R., CREER, S., KNAPP, M., YU, D. W. & DE BRUYN, M. 2014. Environmental DNA for wildlife biology and biodiversity monitoring. *Trends Ecol Evol,* 29**,** 358-367.

BORJA, A., ELLIOTT, M., ANDERSEN, J. H., CARDOSO, A. C., CARSTENSEN, J., FERREIRA, J. G., HEISKANEN, A. S., MARQUES, J. C., NETO, J. M., TEIXEIRA, H., UUSITALO, L., UYARRA, M. C. & ZAMPOUKAS, N. 2013. Good Environmental Status of marine ecosystems: what is it and how do we know when we have attained it? *Mar Pollut Bull,* 76**,** 16-27.

BOYER, S., CRUICKSHANK, R. H. & WRATTEN, S. D. 2015. Faeces of generalist predators as 'biodiversity capsules': A new tool for biodiversity assessment in remote and inaccessible habitats. *Food Webs,* 3**,** 1-6.

BRALEY, M., GOLDSWORTHY, S. D., PAGE, B., STEER, M. & AUSTIN, J. J. 2010. Assessing morphological and DNA-based diet analysis techniques in a

generalist predator, the arrow squid *Nototodarus gouldi*. *Mol Ecol Resour,* 10**,** 466-74.

BROWN, C. J., FULTON, E. A., POSSINGHAM, H. P. & RICHARDSON, A. J. 2011. How long can fisheries management delay action in response to ecosystem and climate change? *Ecological Applications,* 22**,** 298-310.

BROWN, D. S., JARMAN, S. N. & SYMONDSON, W. O. C. 2012. Pyrosequencing of prey DNA in reptile faeces: analysis of earthworm consumption by slow worms. *Molecular Ecology Resources,* 12**,** 259-266.

CASPER, R. M., JARMAN, S. N., DEAGLE, B. E., GALES, N. J. & HINDELL, M. A. 2007a. Detecting prey from DNA in predator scats: A comparison with morphological analysis, using Arctocephalus seals fed a known diet. *Journal of Experimental Marine Biology and Ecology,* 347**,** 144-154.

CASPER, R. M., JARMAN, S. N., GALES, N. J. & HINDELL, M. A. 2007b. Combining DNA and morphological analyses of faecal samples improves insight into trophic interactions: a case study using a generalist predator. *Marine Biology,* 152**,** 815-825.

CLARKE, L. J., BEARD, J. M., SWADLING, K. M. & DEAGLE, B. E. 2017. Effect of marker choice and thermal cycling protocol on zooplankton DNA metabarcoding studies. *Ecol Evol,* 7**,** 873-883.

COWART, D. A., PINHEIRO, M., MOUCHEL, O., MAGUER, M., GRALL, J., MINE, J. & ARNAUD-HAOND, S. 2015. Metabarcoding is powerful yet still blind: a comparative analysis of morphological and molecular surveys of seagrass communities. *PLoS One,* 10**,** e0117562.

DAVIES, C. H., COUGHLAN, A., HALLEGRAEFF, G., AJANI, P., ARMBRECHT, L., ATKINS, N., BONHAM, P., BRETT, S., BRINKMAN, R., BURFORD, M., CLEMENTSON, L., COAD, P., COMAN, F., DAVIES, D., DELA-CRUZ, J., DEVLIN, M., EDGAR, S., ERIKSEN, R., FURNAS, M., HASSLER, C., HILL, D., HOLMES, M., INGLETON, T., JAMESON, I.,

LETERME, S. C., LONBORG, C., MCLAUGHLIN, J., MCENNULTY, F., MCKINNON, A. D., MILLER, M., MURRAY, S., NAYAR, S., PATTEN, R., PRITCHARD, T., PROCTOR, R., PURCELL-MEYERINK, D., RAES, E., RISSIK, D., RUSZCZYK, J., SLOTWINSKI, A., SWADLING, K. M., TATTERSALL, K., THOMPSON, P., THOMSON, P., TONKS, M., TRULL, T. W., URIBE-PALOMINO, J., WAITE, A. M., YAUWENAS, R., ZAMMIT, A. & RICHARDSON, A. J. 2016. A database of marine phytoplankton abundance, biomass and species composition in Australian waters. *Sci Data,* 3**,** 160043.

DEAGLE, B. E., CHIARADIA, A., MCINNES, J. & JARMAN, S. N. 2010. Pyrosequencing faecal DNA to determine diet of little penguins: is what goes in what comes out? *Conservation Genetics,* 11**,** 2039-2048.

DEAGLE, B. E., CLARKE, L. J., KITCHENER, J. A., POLANOWSKI, A. M. & DAVIDSON, A. T. 2017. Genetic monitoring of open ocean biodiversity: An evaluation of DNA metabarcoding for processing continuous plankton recorder samples. *Mol Ecol Resour*.

DEAGLE, B. E., GALES, N. J., EVANS, K., JARMAN, S. N., ROBINSON, S., TREBILCO, R. & HINDELL, M. A. 2007. Studying Seabird Diet through Genetic Analysis of Faeces: A Case Study on Macaroni Penguins (*Eudyptes chrysolophus*). *PLoS ONE,* 2**,** e831.

DEAGLE, B. E., KIRKWOOD, R. & JARMAN, S. N. 2009. Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces. *Molecular Ecology,* 18**,** 2022-2038.

DEAGLE, B. E., TOLLIT, D. J., JARMAN, S. N., HINDELL, M. A., TRITES, A. W. & GALES, N. J. 2005. Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions. *Mol Ecol,* 14**,** 1831-42.

DELPORT, T. C., POWER, M. L., HARCOURT, R. G., WEBSTER, K. N. & TETU, S. G. 2016. Colony Location and Captivity Influence the Gut Microbial

Community Composition of the Australian Sea Lion (*Neophoca cinerea*). *Appl Environ Microbiol,* 82**,** 3440-9.

EMAMI-KHOYI, A., HARTLEY, D. A., PATERSON, A. M., BOREN, L. J., CRUICKSHANK, R. H., ROSS, J. G., MURPHY, E. C. & ELSE, T.-A. 2016. Identifying prey items from New Zealand fur seal (*Arctocephalus forsteri*) faeces using massive parallel sequencing. *Conservation Genetics Resources,* 8**,** 343-352.

FICETOLA, G. F., MIAUD, C., POMPANON, F. & TABERLET, P. Species detection using environmental DNA from water samples. 2008. *Biol. Lett.* 4, 423-425.

FONSECA, V. G., CARVALHO, G. R., SUNG, W., JOHNSON, H. F., POWER, D. M., NEILL, S. P., PACKER, M., BLAXTER, M. L., LAMBSHEAD, P. J., THOMAS, W. K. & CREER, S. 2010. Second-generation environmental sequencing unmasks marine metazoan biodiversity. *Nat Commun,* 1**,** 98.

FOOD AND AGRICULTURAL ORGANIZATION (FAO), *The State of World Fisheries and Aquaculture 2016* (FAO, Rome, 2016).

FOOTE, A., THOMSEN, P., SVEEGAARD, S., WAHLBERG, M., KIELGAST, J., KYHN, L., SALLING, A., GALATIUS, A. & ORLANDO, L. 2012. Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals. *PLoS ONE,* 7**,** e41781.

FORD, M. J., HEMPELMANN, J., HANSON, M. B., AYRES, K. L., BAIRD, R. W., EMMONS, C. K., LUNDIN, J. I., SCHORR, G. S., WASSER, S. K. & PARK, L. K. 2016. Estimation of a Killer Whale (*Orcinus orca*) Population's Diet Using Sequencing Analysis of DNA from Feces. *PLOS ONE,* 11**,** e0144956.

GATTUSO, J. P., MAGNAN, A., BILLÉ, R., CHEUNG, W. W. L., HOWES, E. L., JOOS, F., ALLEMAND, D., BOPP, L., COOLEY, S. R., EAKIN, C. M., HOEGH-GULDBERG, O., KELLY, R. P., PÖRTNER, H. O., ROGERS, A. D., BAXTER, J. M., LAFFOLEY, D., OSBORN, D., RANKOVIC, A.,

ROCHETTE, J., SUMAILA, U. R., TREYER, S. & TURLEY, C. 2015. Contrasting futures for ocean and society from different anthropogenic CO_2 emissions scenarios. *Science,* 349.

GIMMLER, A., KORN, R., DE VARGAS, C., AUDIC, S. & STOECK, T. 2016. The Tara Oceans voyage reveals global diversity and distribution patterns of marine planktonic ciliates. *Sci Rep,* 6**,** 33555.

GRANQUIST, S. M., ESPARZA-SALAS, R., HAUKSSON, E., KARLSSON, O. & ANGERBJÖRN, A. 2018. Fish consumption of harbour seals (*Phoca vitulina*) in north western Iceland assessed by DNA metabarcoding and morphological analysis. *Polar Biology*.

GRIFFITHS, H. J., MEIJERS, A. J. S. & BRACEGIRDLE, T. J. 2017. More losers than winners in a century of future Southern Ocean seafloor warming. *Nature Climate Change,* 7**,** 749-754.

GUARDIOLA, M., URIZ, M. J., TABERLET, P., COISSAC, E., WANGENSTEEN, O. S. & TURON, X. 2015. Deep-Sea, Deep-Sequencing: Metabarcoding Extracellular DNA from Sediments of Marine Canyons. *PLoS One,* 10**,** e0139633.

HARDY, N., BERRY, T., KELAHER, B. P., GOLDSWORTHY, S. D., BUNCE, M., COLEMAN, M. A., GILLANDERS, B. M., CONNELL, S. D., BLEWITT, M. & FIGUEIRA, W. 2017. Assessing the trophic ecology of top predators across a recolonisation frontier using DNA metabarcoding of diets. *Marine Ecology Progress Series,* 573**,** 237-254.

HARVEY, J. B. J., JOHNSON, S. B., FISHER, J. L., PETERSON, W. T. & VRIJENHOEK, R. C. 2017. Comparison of morphological and next generation DNA sequencing methods for assessing zooplankton assemblages. *Journal of Experimental Marine Biology and Ecology,* 487**,** 113-126.

HEATHER, J. M. & CHAIN, B. 2016.The sequence of sequencers: The history of sequencing DNA. *Genomics,* 107:1, 1-8.

HIRAI, J., KURIYAMA, M., ICHIKAWA, T., HIDAKA, K. & TSUDA, A. 2015. A metagenetic approach for revealing community structure of marine planktonic copepods. *Molecular ecology resources,* 15**,** 68-80.

HIRAI, J. & TSUDA, A. 2015. Metagenetic community analysis of epipelagic planktonic copepods in the tropical and subtropical Pacific. *Marine Ecology Progress Series,* 534**,** 65-78.

HUGHES, T. P., KERRY, J. T., BAIRD, A. H., CONNOLLY, S. R., DIETZEL, A., EAKIN, C. M., HERON, S. F., HOEY, A. S., HOOGENBOOM, M. O., LIU, G., MCWILLIAM, M. J., PEARS, R. J., PRATCHETT, M. S., SKIRVING, W. J., STELLA, J. S. & TORDA, G. 2018. Global warming transforms coral reef assemblages. *Nature*. https://doi.org/10.1038/s41586-018-0041-2

IPCC, 2014: Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, SMP 1.2.

ISBELL, F., GONZALEZ, A., LOREAU, M., COWLES, J., DIAZ, S., HECTOR, A., MACE, G. M., WARDLE, D. A., O'CONNOR, M. I., DUFFY, J. E., TURNBULL, L. A., THOMPSON, P. L. & LARIGAUDERIE, A. 2017. Linking the influence and dependence of people on biodiversity across scales. *Nature,* 546**,** 65-72.

JARMAN, S. N., MCINNES, J. C., FAUX, C., POLANOWSKI, A. M., MARTHICK, J., DEAGLE, B. E., SOUTHWELL, C. & EMMERSON, L. 2013. Adelie penguin population diet monitoring by analysis of food DNA in scats. *PLoS One,* 8**,** e82227.

JOLY, S., DAVIES, T. J., ARCHAMBAULT, A., BRUNEAU, A., DERRY, A., KEMBEL, S. W., PERES-NETO, P., VAMOSI, J. & WHEELER, T. A. 2014.

Ecology in the age of DNA barcoding: the resource, the promise and the challenges ahead. *Molecular Ecology Resources,* 14**,** 221-232.

KELLY, P., CLEMENTSON, L., DAVIES, C., CORNEY, S. & SWADLING, K. 2016. Zooplankton responses to increasing sea surface temperatures in the southeastern Australia global marine hotspot. *Estuarine, Coastal and Shelf Science,* 180**,** 242-257.

KELLY, R. P., PORT, J. A., YAMAHARA, K. M. & CROWDER, L. B. 2014. Using Environmental DNA to Census Marine Fishes in a Large Mesocosm. *PLoS ONE,* 9**,** e86175.

KOSOBOKOVA, K. N. & HIRCHE, H.-J. 2016. A seasonal comparison of zooplankton communities in the Kara Sea – With special emphasis on overwintering traits. *Estuarine, Coastal and Shelf Science,* 175**,** 146-156.

LECROQ, B., LEJZEROWICZ, F., BACHAR, D., CHRISTEN, R., ESLING, P., BAERLOCHER, L., ØSTERÅS, M., FARINELLI, L. & PAWLOWSKI, J. 2011. Ultra-deep sequencing of foraminiferal microbarcodes unveils hidden richness of early monothalamous lineages in deep-sea sediments. *Proceedings of the National Academy of Sciences of the United States of America,* 108**,** 13177-13182.

LERAY, M. & KNOWLTON, N. 2016. Censusing marine eukaryotic diversity in the twenty-first century. *Philos Trans R Soc Lond B Biol Sci,* 371.

LINDEQUE, P. K., PARRY, H. E., HARMER, R. A., SOMERFIELD, P. J. & ATKINSON, A. 2013. Next Generation Sequencing Reveals the Hidden Diversity of Zooplankton Assemblages. *PLOS ONE,* 8**,** e81327.

LO, H. K. A. & CHAN, K. Y. K. 2018. Negative effects of microplastic exposure on growth and development of Crepidula onyx. *Environ Pollut,* 233**,** 588-595.

LOTZE, H. K., LENIHAN, H. S., BOURQUE, B. J., BRADBURY, R. H., COOKE, R. G., KAY, M. C., KIDWELL, S. M., KIRBY, M. X., PETERSON, C. H. &

JACKSON, J. B. C. 2006. Depletion, Degradation, and Recovery Potential of Estuaries and Coastal Seas. *Science,* 312**,** 1806.

MACKAS, D. L., BATTEN, S. & TRUDEL, M. 2007. Effects on zooplankton of a warmer ocean: Recent evidence from the Northeast Pacific. *Progress in Oceanography,* 75**,** 223-252.

MACKAS, D. L., GREVE, W., EDWARDS, M., CHIBA, S., TADOKORO, K., ELOIRE, D., MAZZOCCHI, M. G., BATTEN, S., RICHARDSON, A. J., JOHNSON, C., HEAD, E., CONVERSI, A. & PELUSO, T. 2012. Changing zooplankton seasonality in a changing ocean: Comparing time series of zooplankton phenology. *Progress in Oceanography,* 97-100**,** 31-62.

MARKLE, D. F. & FROST, L.-A. 1985. Comparative morphology, seasonality, and a key to planktonic fish eggs from the Nova Scotian shelf. *Canadian Journal of Zoology,* 63**,** 246-257.

MATZ, M. V., TREML, E. A., AGLYAMOVA, G. V. & BAY, L. K. 2018. Potential and limits for rapid genetic adaptation to warming in a Great Barrier Reef coral. *PLoS Genet,* 14**,** e1007220.

MCCAULEY, D. J., PINSKY, M. L., PALUMBI, S. R., ESTES, J. A., JOYCE, F. H. & WARNER, R. R. 2015. Marine defaunation: animal loss in the global ocean. *Science,* 347**,** 1255641.

MCINNES, J. C., ALDERMAN, R., LEA, M. A., RAYMOND, B., DEAGLE, B. E., PHILLIPS, R. A., STANWORTH, A., THOMPSON, D. R., CATRY, P., WEIMERSKIRCH, H., SUAZO, C. G., GRAS, M. & JARMAN, S. N. 2017. High occurrence of jellyfish predation by black-browed and Campbell albatross identified by DNA metabarcoding. *Mol Ecol,* 26**,** 4831-4845.

MCINNES, J. C., EMMERSON, L., SOUTHWELL, C., FAUX, C. & JARMAN, S. N. 2016. Simultaneous DNA-based diet analysis of breeding, non-breeding and chick Adelie penguins. *R Soc Open Sci,* 3**,** 150443.

MIYA, M., SATO, Y., FUKUNAGA, T., SADO, T., POULSEN, J. Y., SATO, K., MINAMOTO, T., YAMAMOTO, S., YAMANAKA, H., ARAKI, H., KONDOH, M. & IWASAKI, W. 2015. MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. *R Soc Open Sci,* 2**,** 150088.

MOKANY, K., RICHARDSON, A. J., POLOCZANSKA, E. S., FERRIER, S. & GROUP, C. C. B. W. 2010. Uniting marine and terrestrial modelling of biodiversity under climate change. *Trends Ecol Evol,* 25**,** 550-1.

MOLINERO, J. C., IBANEZ, F., NIVAL, P., BUECHER, E. & SOUISSI, S. 2005. North Atlantic climate and northwestern Mediterranean plankton variability. *Limnology and Oceanography,* 50**,** 1213-1220.

MORA, C., TITTENSOR, D. P., ADL, S., SIMPSON, G. B. & WORM, B. 2011 How many species are there on earth and in the ocean? *PLoS Biology,* 9(8): e1001127

MORARD, R., LEJZEROWICZ, F., DARLING, K. F., LECROQ-BENNET, B., PEDERSEN, M. W., ORLANDO, L., PAWLOWSKI, J., MULITZA, S., DE VARGAS, C. & KUCERA, M. 2017. Planktonic foraminifera-derived environmental DNA extracted from abyssal sediments preserves patterns of plankton macroecology. *Biogeosciences,* 14**,** 2741.

MURRAY, D. C., BUNCE, M., CANNELL, B. L., OLIVER, R., HOUSTON, J., WHITE, N. E., BARRERO, R. A., BELLGARD, M. I. & HAILE, J. 2011. DNA-Based Faecal Dietary Analysis: A Comparison of qPCR and High Throughput Sequencing Approaches. *Plos One,* 6.

O'BRIEN, B. S., MELLO, K., LITTERER, A. & DIJKSTRA, J. A. 2018. Seaweed structure shapes trophic interactions: A case study using a mid-trophic level fish species. *Journal of Experimental Marine Biology and Ecology,* 506**,** 1-8.

O'RORKE, R., LAVERY, S. & JEFFS, A. 2012. PCR enrichment techniques to identify the diet of predators. *Molecular Ecology Resources,* 12**,** 5-17.

O'CONNOR, S., ONO, R. & CLARKSON, C. 2011. Pelagic Fishing at 42,000 Years Before the Present and the Maritime Skills of Modern Humans. *Science,* 334**,** 1117.

O'RORKE, R., LAVERY, S. D., WANG, M., NODDER, S. D. & JEFFS, A. G. 2013. Determining the diet of larvae of the red rock lobster (*Jasus edwardsii*) using high-throughput DNA sequencing techniques. *Marine Biology,* 161**,** 551-563.

OLIVER, E. C. J., DONAT, M. G., BURROWS, M. T., MOORE, P. J., SMALE, D. A., ALEXANDER, L. V., BENTHUYSEN, J. A., FENG, M., SEN GUPTA, A., HOBDAY, A. J., HOLBROOK, N. J., PERKINS-KIRKPATRICK, S. E., SCANNELL, H. A., STRAUB, S. C. & WERNBERG, T. 2018. Longer and more frequent marine heatwaves over the past century. *Nat Commun,* 9**,** 1324.

OSTERHAGE, D., POGONOSKI, J. J., APPLEYARD, S. A. & WHITE, W. T. 2016. Integrated Taxonomy Reveals Hidden Diversity in Northern Australian Fishes: A New Species of Seamoth (Genus *Pegasus*). *PLOS ONE,* 11**,** e0149415.

PAWLOWSKI, J., ESLING, P., LEJZEROWICZ, F., CEDHAGEN, T. & WILDING, T. A. 2014. Environmental monitoring through protist next-generation sequencing metabarcoding: assessing the impact of fish farming on benthic foraminifera communities. *Mol Ecol Resour,* 14**,** 1129-40.

PETERS, K. J., OPHELKELLER, K., BOTT, N. J., DEAGLE, B. E., JARMAN, S. N. & GOLDSWORTHY, S. D. 2014. Fine-scale diet of the Australian sea lion (*Neophoca cinerea*) using DNA-based analysis of faeces. *Marine Ecology***,** 1-21.

PETERS, K. J., OPHELKELLER, K., HERDINA, BOTT, N. J. & GOLDSWORTHY, S. D. 2015. PCR-based techniques to determine diet of the Australian sea lion (*Neophoca cinerea*): a comparison with morphological analysis. *Marine Ecology,* 36**,** 1428-1439.

PIMM, S. L., JENKINS, C. N., ABELL, R., BROOKS, T. M., GITTLEMAN, J. L., JOPPA, L. N., RAVEN, P. H., ROBERTS, C. M. & SEXTON, J. O. 2014.

The biodiversity of species and their rates of extinction, distribution, and protection. *Science,* 344.

POCHON, X., BOTT, N. J., SMITH, K. F. & WOOD, S. A. 2013. Evaluating Detection Limits of Next-Generation Sequencing for the Surveillance and Monitoring of International Marine Pests. *PLOS ONE,* 8**,** e73935.

POLOCZANSKA, E. S., BROWN, C. J., SYDEMAN, W. J., KIESSLING, W., SCHOEMAN, D. S., MOORE, P. J., BRANDER, K., BRUNO, J. F., BUCKLEY, L. B., BURROWS, M. T., DUARTE, C. M., HALPERN, B. S., HOLDING, J., KAPPEL, C. V., O'CONNOR, M. I., PANDOLFI, J. M., PARMESAN, C., SCHWING, F., THOMPSON, S. A. & RICHARDSON, A. J. 2013. Global imprint of climate change on marine life. *Nature Climate Change,* 3**,** 919-925.

POMPANON, F., DEAGLE, B. E., SYMONDSON, W. O., BROWN, D. S., JARMAN, S. N. & TABERLET, P. 2012. Who is eating what: diet assessment using next generation sequencing. *Mol Ecol,* 21**,** 1931-50.

PRIETO-DAVÓ, A., VILLARREAL-GÓMEZ, L. J., FORSCHNER-DANCAUSE, S., BULL, A. T., STACH, J. E. M., SMITH, D. C., ROWLEY, D. C. & JENSEN, P. R. 2013. Targeted search for actinomycetes from nearshore and deep-sea marine sediments. *FEMS Microbiology Ecology,* 84**,** 510-518.

PURCELL, J. E. 2012. Jellyfish and ctenophore blooms coincide with human proliferations and environmental perturbations. *Annual Review of Marine Science,* 4**,** 209-235.

RAKHESH, M., RAMAN, A. V. & SUDARSAN, D. 2006. Discriminating zooplankton assemblages in neritic and oceanic waters: A case for the northeast coast of India, Bay of Bengal. *Marine Environmental Research,* 61**,** 93-109.

RATNASINGHAM, S. & HEBERT PAUL D, N. 2007. BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). *Molecular Ecology Notes,* 7**,** 355-364.

RICE, E. & STEWART, G. 2016. Decadal changes in zooplankton abundance and phenology of Long Island Sound reflect interacting changes in temperature and community composition. *Mar Environ Res,* 120**,** 154-165.

RICHARDSON, A. 2009. Plankton and climate. *Elements of Physical Oceanography: A derivative of the Encyclopedia of Ocean Sciences***,** 397.

RICHARDSON, A. J., BROWN, C. J., BRANDER, K., BRUNO, J. F., BUCKLEY, L., BURROWS, M. T., DUARTE, C. M., HALPERN, B. S., HOEGH-GULDBERG, O., HOLDING, J., KAPPEL, C. V., KIESSLING, W., MOORE, P. J., O'CONNOR, M. I., PANDOLFI, J. M., PARMESAN, C., SCHOEMAN, D. S., SCHWING, F., SYDEMAN, W. J. & POLOCZANSKA, E. S. 2012. Climate change and marine life. *Biol Lett,* 8**,** 907-9.

SAMANTA, B. & BHADURY, P. 2014. Analysis of diversity of chromophytic phytoplankton in a mangrove ecosystem using rbcL gene sequencing. *J Phycol,* 50**,** 328-40.

SANGER, F., NICKLEN, S. & COULSON, A. R. 1977. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National academy of Sciences,* 74:12, 5463-5467.

SCHNACK-SCHIEL, S. B., MICHELS, J., MIZDALSKI, E., SCHODLOK, M. P. & SCHRÖDER, M. 2008. Composition and community structure of zooplankton in the sea ice-covered western Weddell Sea in spring 2004—with emphasis on calanoid copepods. *Deep Sea Research Part II: Topical Studies in Oceanography,* 55**,** 1040-1055.

SCHNACK-SCHIEL, S. B., MIZDALSKI, E. & CORNILS, A. 2010. Copepod abundance and species composition in the Eastern subtropical/tropical Atlantic. *Deep Sea Research Part II: Topical Studies in Oceanography,* 57**,** 2064-2075.

SIGSGAARD, E. E., NIELSEN, I. B., BACH, S. S., LORENZEN, E. D., ROBINSON, D. P., KNUDSEN, S. W., PEDERSEN, M. W., JAIDAH, M.

A., ORLANDO, L., WILLERSLEV, E., MØLLER, P. R. & THOMSEN, P. F. 2016. Population characteristics of a large whale shark aggregation inferred from seawater environmental DNA. *Nature Ecology & Evolution,* 1**,** 0004.

SIGSGAARD, E. E., NIELSEN, I. B., CARL, H., KRAG, M. A., KNUDSEN, S. W., XING, Y., HOLM-HANSEN, T. H., MØLLER, P. R. & THOMSEN, P. F. 2017 Seawater environmental DNA reflects seasonality of a coastal fish community. *Mar Biol.* 164: 128

SIMPSON, T. J., DIAS, P. J., SNOW, M., MUNOZ, J. & BERRY, T. 2016. Real-time PCR detection of *Didemnum perlucidum* (Monniot, 1983) and *Didemnum vexillum* (Kott, 2002) in an applied routine marine biosecurity context. *Mol Ecol Resour*.

STAT, M., HUGGETT, M. J., BERNASCONI, R., DIBATTISTA, J. D., BERRY, T. E., NEWMAN, S. J., HARVEY, E. S. & BUNCE, M. 2017. Ecosystem biomonitoring with eDNA: metabarcoding across the tree of life in a tropical marine environment. *Sci Rep,* 7**,** 12240.

STOECKLE, M. Y., SOBOLEVA, L. & CHARLOP-POWERS, Z. 2017. Aquatic environmental DNA detects seasonal fish abundance and habitat preference in an urban estuary. *PLOS ONE,* 12**,** e0175186.

SU, M., LIU, H., LIANG, X., GUI, L. & ZHANG, J. 2017. Dietary Analysis of Marine Fish Species: Enhancing the Detection of Prey-Specific DNA Sequences via High-Throughput Sequencing Using Blocking Primers. *Estuaries and Coasts,* 41**,** 560-571.

TABERLET, P., BONIN, A., ZINGER, L. & COISSAC, E. 2018. Environmental DNA for biodiversity research and monitoring. *Introduction to environmental DNA.* Oxford University Press. Chapter 1

TABERLET, P., COISSAC, E., HAJIBABAEI, M. & RIESEBERG, L. H. 2012. Environmental DNA. *Molecular Ecology,* 21**,** 1789-1793.

THOMAS, A. C., JARMAN, S. N., HAMAN, K. H., TRITES, A. W. & DEAGLE, B. E. 2014. Improving accuracy of DNA diet estimates using food tissue control materials and an evaluation of proxies for digestion bias. *Mol Ecol,* 23**,** 3706-18.

THOMSEN, P., KIELGAST, J., IVERSEN, L., MOLLER, P., RASMUSSEN, M., MÃ¸LLER, P. & WILLERSLEV, E. 2012. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. *PLoS ONE,* 7**,** e41732.

THOMSEN, P. F. & WILLERSLEV, E. 2015. Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation,* 183**,** 4-18.

TURNER, C. R., UY, K. L. & EVERHART, R. C. 2015. Fish environmental DNA is more concentrated in aquatic sediments than surface water. *Biological Conservation,* 183**,** 93-102.

VACHER, C., TAMADDONI-NEZHAD, A., KAMENOVA, S., PEYRARD, N., MOALIC, Y., SABBADIN, R., SCHWALLER, L., CHIQUET, J., SMITH, M. A. & VALLANCE, J. 2016. Learning ecological networks from next-generation sequencing data. *Advances in Ecological Research.* Elsevier.

VESTHEIM, H. & JARMAN, S. N. 2008. Blocking primers to enhance PCR amplification of rare sequences in mixed samples - a case study on prey DNA in Antarctic krill stomachs. *Front Zool,* 5**,** 12.

VON AMMON, U., WOOD, S. A., LAROCHE, O., ZAIKO, A., TAIT, L., LAVERY, S., INGLIS, G. & POCHON, P. 2018 The impact of artificial surfaces on marine bacterial and eukaryotic biofouling assemblages: A high-throughput sequencing analysis. *Marine Environmental Research,* 133, 57-66.

WATSON, J. D. & CRICK, F. H. C. 1953. Molecular structure of Nucleic Acids. *Nature,* 171, 737-738.

WERNBERG, T., BENNETT, S., BABCOCK, R. C., DE BETTIGNIES, T., CURE, K., DEPCZYNSKI, M., DUFOIS, F., FROMONT, J., FULTON, C. J.,

HOVEY, R. K., HARVEY, E. S., HOLMES, T. H., KENDRICK, G. A., RADFORD, B., SANTANA-GARCON, J., SAUNDERS, B. J., SMALE, D. A., THOMSEN, M. S., TUCKETT, C. A., TUYA, F., VANDERKLIFT, M. A. & WILSON, S. 2016. Climate-driven regime shift of a temperate marine ecosystem. *Science,* 353**,** 169.

WILLIAMS, R. 1984. Zooplankton of the Bristol Channel and Severn Estuary. *Marine Pollution Bulletin,* 15**,** 66-70.

ZHAN, A., HULÁK, M., SYLVESTER, F., HUANG, X., ADEBAYO, A. A., ABBOTT, C. L., ADAMOWICZ, S. J., HEATH, D. D., CRISTESCU, M. E., MACISAAC, H. J. & POND, S. K. 2013. High sensitivity of 454 pyrosequencing for detection of rare species in aquatic communities. *Methods in Ecology and Evolution,* 4**,** 558-565.

Chapter – Two

DNA metabarcoding for diet analysis and biodiversity: A case study using the endangered Australian sea lion (*Neophoca cinerea*)

The Australian sea lion preys,
on demersal fish, crustaceans and rays -
hauls out on the beach, to soak up the sun
then sighs and goes back to the waves

'Sea lion days' – Tina E Berry

2.1 Prelude

WHILE Chapter One introduces the history of and reasons behind the evolution of eDNA and metabarcoding, this chapter explores and refines the use next generation sequencing and metabarcoding of DNA to characterise the diet of an endangered species and its ability to sample the biodiversity of its habitat[1].

The Australian sea lion (*Neophoca cinerea*) is an endangered, apex predator that is endemic to the southwest of Australia. Its diet is limited by a combination of its foraging range and the prey found within that range. Hence each sea lion consumes samples of its own surrounding biodiversity. Conventional dietary studies are mostly either observational or morphological in nature. However, these methods can be problematic. Observational methods are logistically challenging in a marine environment and morphological analysis traditionally examines gut contents from necroscopies or scat. Gastrointestinal samples from sea lions are quite degraded, limiting the information that can be drawn from them.

Sea lion scat is readily collected off the beach from habitual sea lion haul out sites. This provides a convenient substrate for an eDNA evaluation with minimal disturbance to the animal. Suitable extraction methods were investigated and several barcoding assays were explored during the study, including three novel sets. The new assays were

[1] This study was an extension of research undertaken during my honours year. The reuse of the data from that study was limited to the confirmation of the origin of each scat as sea lion (Mam 16S) and the detection of the preyed birds (Bird 12S). The continuation involved the testing of new assays (Plank COI and S_Ceph 16S) and the development of more (Crust and Ceph 16S) The Fish 16S data was regenerated entirely using the new sequencing platform (Illumina's MiSeq). – Berry, T.E. (2013) 'Development of molecular tools for dietary analysis: a tail end look at Western Australia's aquatic ecosystems', BS (Molecular Biology), Murdoch University, Western Australia.

developed to target Fish, Cephalopods and Crustaceans. The aim of the project was to assess the conservational value of the method and determine what information could be gathered about the diet of the pinniped, the biodiversity of its environment and any impact it might have on commercial fisheries.

The results of this study are published in *Ecology and Evolution* (Berry et al (2017) Vol. 7(14), pg. 5435-5453); unique sequence data from the study are uploaded to Data Dryad (https://doi.org/10.5061/dryad.rd748). The following chapter reproduces the paper as published with the exception of formatting and referencing. An as published version is available in the Appendix.

2.1.1 Acknowledgements

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2.1.2 Author Contributions

TB was involved with all aspects of the design and implementation of the experiment and the production of the manuscript. MC provided valuable assistance in the laboratory. LB and MB conceived the idea for the project and SO collected the samples. DM aided with data filtration and designed an assay. AG created the figures. AR instigated the statistical analysis. All authors contributed their expertise to edit and refine the paper but this was particularly so for MB and MS.

2.1.3 Author Declarations

Faecal samples were collected under a Department of Parks and Wildlife (DPaW) permit (number SF009371) as well as in accordance with Victoria University's, Melbourne ethics committee's approval: AEETH24/11 and AEC_2013_32 granted by Curtin University, Perth.

The Authors declare they have no competing interests.

DNA metabarcoding for diet analysis and biodiversity: a case study using the endangered Australian sea lion (*Neophoca cinerea)*

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2.2 Abstract

THE analysis of apex predator diet has the ability to deliver valuable insights into ecosystem health and the potential impacts a predator might have on commercially relevant species. The Australian sea lion (*Neophoca cinerea*) is an endemic apex predator and one of the world's most endangered pinnipeds. Given that prey availability is vital to the survival of top predators, this study set out to understand what dietary information DNA metabarcoding could yield from 36 sea lion scats collected across 1,500km of its distribution in southwest Western Australia. A combination of PCR assays were designed to target a variety of potential sea lion prey, including mammals, fish, crustaceans, cephalopods and birds. Over 1.2 million metabarcodes identified six classes from three phyla, together representing over 80 taxa. The results confirm that the Australian sea lion is a wide-ranging opportunistic predator that consumes an array of mainly demersal fauna. Further, the important commercial species *Sepioteuthis australis* (southern calamari squid) and *Panulirus cygnus* (western rock lobster) were detected, but were present in < 25% of samples. Some of the taxa identified, such as fish, sharks and rays, clarify previous knowledge of sea lion prey, and some, such as eel taxa and two gastropod species, represent new dietary insights. Even with modest sample sizes a spatial analysis of taxa and operational taxonomic units found within the scat show significant differences in diet between many of the sample locations and identifies the primary taxa that are driving this variance. This study provides new insights into the diet of this endangered predator and confirms the efficacy of DNA metabarcoding of scat as a noninvasive tool to more broadly define regional biodiversity.

2.3 Introduction

THE majority of marine mammals are generalist predators that consume prey from many trophic levels (Casper et al., 2007) and therefore potentially influence the community structure of marine environments. As such, the analysis of their diet can provide the opportunity for a comprehensive assessment of the biodiversity present in marine ecosystems (Casper et al., 2007, Boyer et al., 2015).

The Australian sea lion (Figure 2.1) is one of the rarest sea lion species in the world (Hesp et al., 2012) and Australia's only endemic pinniped species (Kirkwood and Goldsworthy, 2013, Ling, 1992). In 2015, there were an estimated 12,290-13,090 individuals remaining in the wild and of these only 16% are found in Western Australia (Goldsworthy, 2015). Australian sea lions are distributed between the Abrolhos Islands in Western Australia and The Pages in South Australia (Ling, 1992), with mostly small and widely scattered colonies, at both remote (Goldsworthy et al., 2009, Goldsworthy, 2015) and near metropolitan areas (Osterrieder et al., 2015, Osterrieder et al., 2016). Despite several dietary studies (Gales and Cheal, 1992, Casper et al., 2007, Peters et al., 2014, Ling, 1992, Kirkwood and Goldsworthy, 2013), much of what this apex predator targets remains poorly defined due to the well-recognized limits of morphological identification of scat material and/or behavioural studies (Kirkwood and Goldsworthy, 2013). Such studies suggest that the Australian sea lion is a largely nocturnal forager (Kirkwood and Goldsworthy, 2013), although studies of females and pups from Kangaroo Island, South Australia, suggest that their foraging does not follow a diurnal pattern (Costa and Gales, 2003). These previous studies have also shown that sea lions prey mainly on benthic species of fish, sharks, rays, cephalopods and crustaceans (Kirkwood and Goldsworthy, 2013); however, other evidence also suggests that they prey on rock lobster, swimming crabs, shark eggs and penguins (McIntosh et al., 2006). A more recent molecular approach used bacterial cloning and Sanger sequencing of DNA to identify 23 fish and five cephalopod taxa from the scats of 12 female sea lions from two colonies in South Australia (Peters et al., 2014), finding several new taxa upon which sea lions prey.

FIGURE 2.1 The Australian sea lion (*Neophoca cinerea*) at Seal Island, Shoalwater Bay, Western Australia.

Observational studies on diet in marine systems can be logistically difficult to conduct and expensive. This is especially true where the animal in question is fast, feeds underwater, and has a large foraging range, as is the case with sea lions (Kirkwood and Goldsworthy, 2013). These problems can be compounded when the study animal is reclusive and/or hunts nocturnally (such as sea lions). In contrast, the collection of sea lion scat is relatively easy as it can be collected by hand from the beaches of known sea lion haul out points. However, morphological analysis of scat has several complications. Firstly, dietary identification relies heavily on the presence of prey remnants and prey that is relatively undigested may be over represented while highly digested prey may be missed (Shehzad et al., 2012a, Brown et al., 2012, Boyer et al., 2015). Therefore, fleshy or gelatinous targets are unlikely to be detected. In the case of the sea lion, smaller cephalopod beaks and fish otoliths digest completely, or are unrecognizable, once they have passed through the digestive tract (Gales and Cheal, 1992, Peters et al., 2015). This issue is partially attributed to the grinding action of large gastroliths found in the sea lions' stomach (McIntosh et al., 2006). Gastroliths are large stones that can measure up to approximately 7 cm in diameter, and are swallowed by sea lions as ballast (Kirkwood and Goldsworthy, 2013). Secondly, some potential prey species, such as crustaceans, are morphologically similar to one another (Radulovici et al., 2009), making identification of their remains taxonomically challenging. Further, due to the increased rate of survival of cephalopod beaks in comparison to fish otoliths, reliance on morphological analysis of sea lion scat for dietary analysis can lead to an underestimation of fish but an overestimation of cephalopods consumed (Gales and Cheal, 1992, Peters et al., 2015).

Recent advances in DNA sequencing (and analyzing) environmental samples have enhanced the capacity to identify constituents of fecal material (Pompanon et al., 2012). The use of standard DNA barcodes, PCR, and reference sequence databases facilitate the analysis of prey taxa (or their DNA) that survive in fecal material. DNA metabarcoding approaches (employing next generation sequencing, NGS), where complex mixtures of DNA are extracted and sequenced in parallel, have been successfully applied to several fecal dietary studies with promising results (Hibert et al., 2013, Quemere et al., 2013, Shehzad et al., 2012b, Murray et al., 2011, Berry et al., 2015). One of the first studies

to exploit DNA metabarcoding, investigated the diet of the Australian fur seal (*Arctocephalus pusillus*; (Deagle et al., 2009) and, in a more recent study, the diets of both the Australian (*A. pusillus doriferus*) and long-nosed fur seals (*A. forsterii)* were compared (Hardy et al., 2017). To date, no metabarcoding studies exist to explore the Australian sea lion diet but recently a gut microbiome study was conducted on both wild and captive populations (Delport et al., 2016). It is suggested that this type of study could, in future, be combined with a dietary analysis to determine what impact diet has on gut flora.

Using DNA metabarcoding on 36 scat samples, this study seeks to develop and apply multi-gene metabarcoding assays for the analysis of the diet of the Australian sea lion. The purpose of the results are three fold: (1) to determine the effectiveness of DNA metabarcoding for the dietary analysis of the Australian sea lion and the marine biodiversity that supports them, (2) to assess the predation of commercially valued fishes, and (3) to establish whether this type of study could be used to detect spatial changes in sea lion prey across the southwest of Australia. Importantly, as the Australian sea lion is an endangered species (IUCN Red List; (Goldsworthy, 2015), it is of value to develop a holistic picture of what dietary options these apex predators exploit and how these differ spatially and temporally.

2.4 Methods

2.4.1 Sample collection

In total, 36 fecal samples were collected in sterile containers from islands across five collection sites that stretch 1,500 km of the southwest coast of Western Australia (Figure 2.2; for greater detail about dates and sites see Table A2.1). Scat samples were preserved and stored at -20°C.

2.4.2 Metabarcoding Assay Design

Several PCR assays were designed and/or optimized for use in DNA metabarcoding workflows including the Fish 16S, Ceph 16S and the Crust 16S assays (Table 2.1). All primer sets flank hypervariable regions of the 16S rRNA gene and were designed and tested *in silico* using reference sequences obtained from GenBank. For the Ceph 16S assay, 27 16S sequences from different Western Australian cephalopods were analyzed *in silico* to identify short conserved areas of the target gene, which will amplify degraded DNA. Similarly, the Crust 16S assay was designed using 13 16S crustacean sequences including crayfish, crab and prawn species. All newly designed primers were tested against sea lion sequences to ensure no significant amplification of host DNA. To determine the efficacy of the assays, amplifications were optimized on single-source reference tissue including some crustaceans, a cephalopod and several species of fish (Table A2.2).

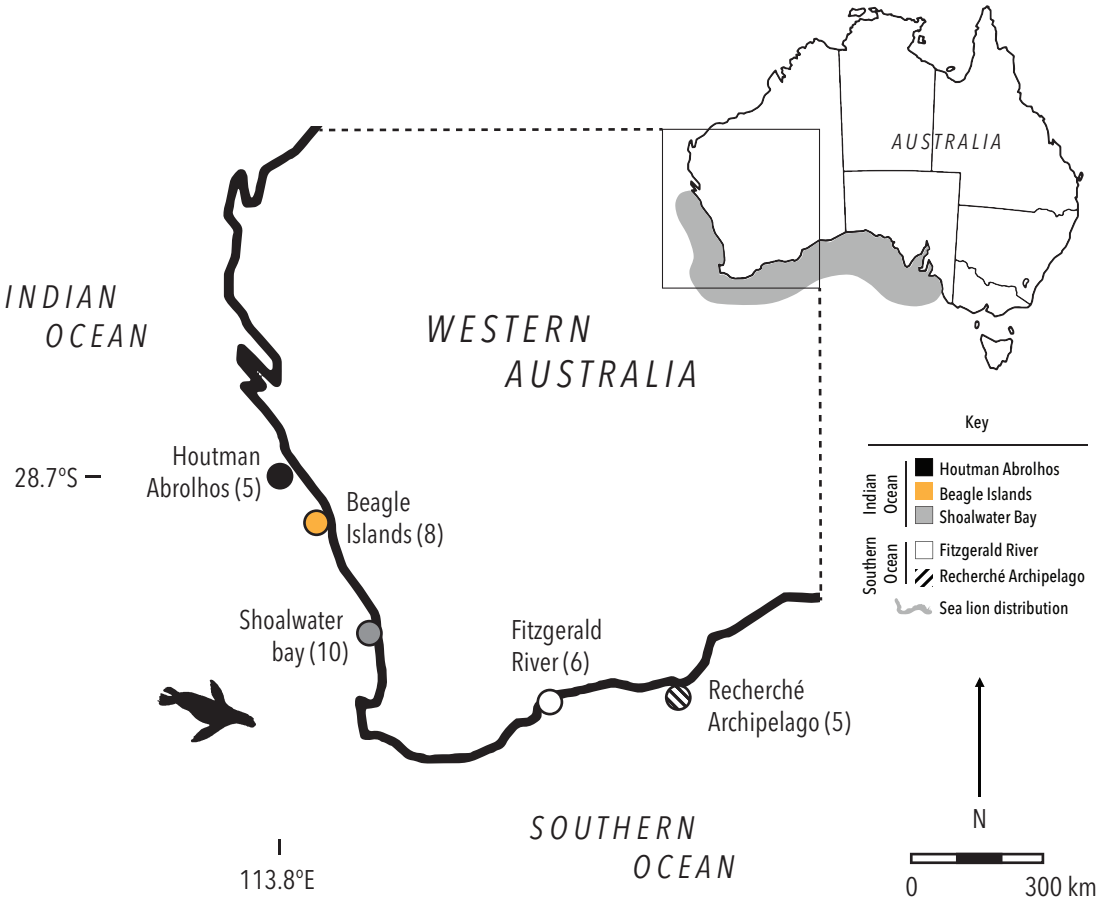

FIGURE 2.2 Sampling sites for metabarcoding study; Map of Australia, with inset showing southern Western Australian sampling sites (number of scats in brackets). The shaded areas denote the range of the Australian sea lion across Australia and within Western Australia.

TABLE 2.1 Metabarcoding PCR assays and the primer sets used for dietary analysis of *Neophoca cinerea* scat.

PCR assay	Primer set used	Target Taxa	Gene	Primer sequence	Amplicon length (bp)	Reference	Assay T_m (°C)
Bird 12S	12Sa (F) 12Sh (R)	Birds	12S rRNA	5′ CTGGGATTAGATACCCCACTAT 3′ 5′ CCTTGACCTGTCTTGTTAGC 3′	~230	Cooper (1994)	57
Fish 16S	Fish16sF/D 16s2R (degenerate)	Fish	16S rRNA	5′ GACCCTATGGAGCTTTAGAC 3′ 5′ CGCTGTTATCCCTADRGTAACT 3′	~200	F- This study R-Deagle et al. (2007)	54
Plank COI	(Plank)Minibar-Mod-F (Plank)Minibar-Mod-R	Plankton	COI	5′ TCCACTAATCACAAAGAYATYGGYAC 3′ 5′ AGAAAATCATAATRAANGCRTGNGC 3′	~127	(Berry et al., 2015)	52
Ceph 16S	Ceph16S1_F(deg) Ceph16SR_Short	Cephalopods	16S rRNA	5′ GACGAGAAGACCCTADTGAGC 3′ 5′ CCAACATCGAGGTCGCAATC 3′	~200	F- Modified from Peters et al. (2014) R- This study	55
Crust 16S	Crust16S_F(short) Crust16S_R(short)	Crustaceans	16S rRNA	5′ GGGACGATAAGACCCTATA 3′ 5′ ATTACGCTGTTATCCCTAAAG 3′	~170	This study	51
Mam 16S	16Smam1 (F) 16Smam2 (R)	Mammals	16S rRNA	5′ CGGTTGGGGTGACCTCGGA 3′ 5′ GCTGTTATCCCTAGGGTAACT 3′	~90	Taylor (1996)	57
S_Ceph 16S	S_Cephalopoda_F S_Cephalopoda_R	Cephalopods	16S rRNA	5′ GCTRGAATGAATGGTTTGAC 3′ 5′ TCAWTAGGGTCTTCTCGTCC 3′	~70	Peters et al. (2014)	50

"F" refers to the forward primer; "R" refers to the reverse primer.

2.4.3 DNA extraction and quantification

Scats were sub-sampled (100-290 mg) and the DNA was extracted using the QIAmp Stool Mini Kit (Qiagen, CA, USA), following the manufacturer's instructions but using an overnight digestion at 55°C, 0.5 x InhibitEX tablet, and eluting in 50 µL of AE Buffer. Extracts were diluted (1/5 and 1/20) in order to assess assay response, and amplification efficiency and inhibition using quantitative PCR (qPCR). All qPCR reactions were carried out in 25 µL consisting of final concentrations of: 1 x Taq Gold buffer (Applied Biosystems [ABI], USA), 2 mM $MgCl_2$ (ABI, USA), 0.4 mg/mL BSA (Fisher Biotec, Australia), 0.25 mM dNTPs (Astral Scientific, Australia), 0.4 µM each of forward and reverse primers (Integrated DNA Technologies, Australia), 0.6 µL of 1/10,000 SYBR Green dye (Life Technologies, USA), 1 U of Taq polymerase Gold (ABI, USA), 2 µL of DNA, and made to volume with ultrapure water.

Each qPCR was run on a Step-ONE qPCR thermocycler (ABI, USA) under the following conditions: 95°C for 5 min, followed by 50 cycles of 95°C for 30 s, 54-58°C for 30 s (the annealing temperature of each primer set is represented in Table 2.1) and 72°C for 45 s and a final extension of 10 min at 72°C. Where qPCR of an extract produced results in response to an assay, the DNA dilution with the highest relative proportion of starting template that showed uninhibited amplification (determined by qPCR C_T values) was selected for subsequent metabarcoding using assay specific fusion tagged primers (The number of PCR – positive samples from each site and assay are shown in Table A2.3). The optimization of input DNA in amplicon sequencing workflows has been shown previously to benefit the sensitivity, reproducibility and quality of metabarcoding data (Murray et al., 2015).

2.4.4 Library build and sequencing

Fusion tagged primers are gene specific primers which also incorporate MID (Multiplex IDentifier) tags of six to eight base pairs in length, and the appropriate Illumina/454 adaptor sequences. Unique combinations of these MID tags were assigned to each individual DNA extract to allow for the assignment of sequences to a sample post sequencing of pooled samples. To minimize cross contamination (in highly sensitive

NGS workflows) no primer-MID combination had been previously used, nor were combinations reused. Fusion PCR reactions were performed on DNA extracts (appropriate dilution determined by qPCR) in duplicate and thermocycling conditions were used as described above. Tagged amplicons were purified using the Agencourt™ AMPure™ (Beckman Coulter Genomics, MA, USA) XP Bead PCR Purification kit as per the manufacturer's instructions, with the addition of a five-minute incubation prior to elution at room temperature. The size and concentration of amplicons were estimated by electrophoresis on a 2% agarose gel stained with GelRed (Fisher Biotec, Australia), followed by visualization under UV light using a BioRad transilluminator**.**

Amplicons were combined in approximately equimolar concentrations to produce a single DNA library of all extracts for sequencing. The resultant library was purified as described above and quantified alongside a set of standard synthetic oligonucleotides of known molarity (Bunce et al., 2012) via qPCR, prior to sequencing (95°C for 5 min followed by 40 cycles of 95°C for 30 s and 60°C for 45 s). For the Mam 16S and Bird 12S assays, all sequencing was performed on Roche's 454 GS Junior (Lib A chemistry). For the remainder of the assays, sequencing was achieved using Illumina's MiSeq® (300 cycle, version 2 reagent kit and Nano flow cell), following manufacturers protocols.

2.4.5 Data filtering and bioinformatics

Sequences were assigned to samples based on their MID tag using Geneious v.R8 (Kearse et al., 2012). As a method for quality control, only amplicons that contained a 100% nucleotide match to the MID, gene-specific primer and sequencing adapter regions were kept for further analysis (the number of reads passing this filter for each assay and per site are shown in Table A2.4). Adaptor/primer regions were removed and the remaining amplicons were filtered using USEARCH's fastq filter with a maximum error of 0.5 (Edgar, 2010). The sequences were then separated into groups of unique sequences (this data is available for download on Data Dryad, https://doi.org/10.5061/dryad.rd748). Groups with sequence numbers of <1% of the total number of unique sequences detected in the sample were discarded in order to remove low-abundant and potentially erroneous sequences (i.e. sequencing error and

chimeras). Amplicons passing quality filtering were searched against the National Center for Biotechnology Information's (NCBI) GenBank nucleotide database (April 29 2015; (Benson et al., 2012) using BLASTn (Basic Local Alignment Search Tool; (Altschul et al., 1990) with the default parameters and a reward of 1. BLAST output files were imported into MEGAN (METaGenome ANalyzer; (Huson et al., 2011) and visualized against the NCBI taxonomic framework using the LCA parameters: reporting of all reads, min bitscore 65.0, and reports limited to top 5% matches. Assignment of sequences to taxa was only considered where a match was made across the entire length of the query. Where further information was required regarding the habitat and commercialization of a species, the Atlas of Living Australia (2016) and FishBase (Froese, 2016) were consulted (the number of reads assigned for each site and assay, are shown in Table A2.4).

2.4.6 Operational taxonomic unit analysis

The operational taxonomic unit (OTU) analysis was performed using USEARCH (Edgar, 2010). Sequences were grouped into clusters (OTUs) using a 97% similarity threshold. The process also removed any chimeras, as well as clusters with a sequence abundance below 0.75% of the total number of unique sequences detected within the sample. Empirically these thresholds retained the sensitivity of the metabarcoding assays but removed low abundance OTUs that may be sequencing/PCR artefacts.

2.4.7 Statistical analysis

Despite the modest number of samples and sites, and the issues involving sampling times of the year, a statistical analysis was explored. Accordingly a Jaccard dissimilarity index of presence/absence data, were performed in R (R Core 2015) using the Vegan (Oksanen et al., 2016) and labdsv (Indval; (Roberts, 2016)) packages. A nested non-parametric (permutational) multivariate analysis of variance (adonis) was used to determine whether sea lion diet differed significantly between the five sampling areas nested within the Southern and Indian Oceans. A pairwise adonis with Holm correction (McLaughlin and Sainani, 2014) was also undertaken to ascertain the contribution of each site to the differences seen. The relationship of sampling sites was visualized using a

nonmetric multidimensional scaling (nMDS). Finally, an estimate of indicator value (indval) was calculated to determine which taxa significantly influenced any differences observed in sea lion diet between oceans, and among sites within each ocean. While it was tempting to investigate the relative abundance of NGS reads (within a PCR assay) the value of extracting quantitative data is questionable and unreliable. This is due to the variability in digestion rate and prey biomass, primer bias, mitochondrial molarity and lack of conversion factors (Thomas et al., 2014, Deagle et al., 2005). Accordingly, analyses were restricted to presence/absence data.

2.5 Results and discussion

2.5.1 Overview of the results

The Mam 16S assay was used first to test whether the scat collected originated from an Australian sea lion. The remainder of the metabarcoding assays were used to determine the prey diversity found within the sea lion scats from each site. The taxa found belonged to six classes (Figure 2.3) from three phyla, representing over 20 orders and almost 40 families of prey.

The Mam16S assay confirmed that 34 of the 36 beach-collected samples originated from Australian sea lions (100% matches to reference *N. cinerea* DNA sequences), many of these were later confirmed by the Plank COI assay. Of the two negative samples, one contained large amounts of human DNA while the other contained DNA that was amplified by the bird-specific primers, potentially identifying the originator of the scat as *Pellicanus conspicillatus* (Australian pelican). These two samples were excluded from further analysis.

The non-mammalian metabarcoding assays were designed to characterize fish, crustacean and cephalopod prey in these environmental samples. It is suggested that these assays will be useful for future metabarcoding studies on marine substrates such as scat, water, sediment, plankton tows and gut contents.

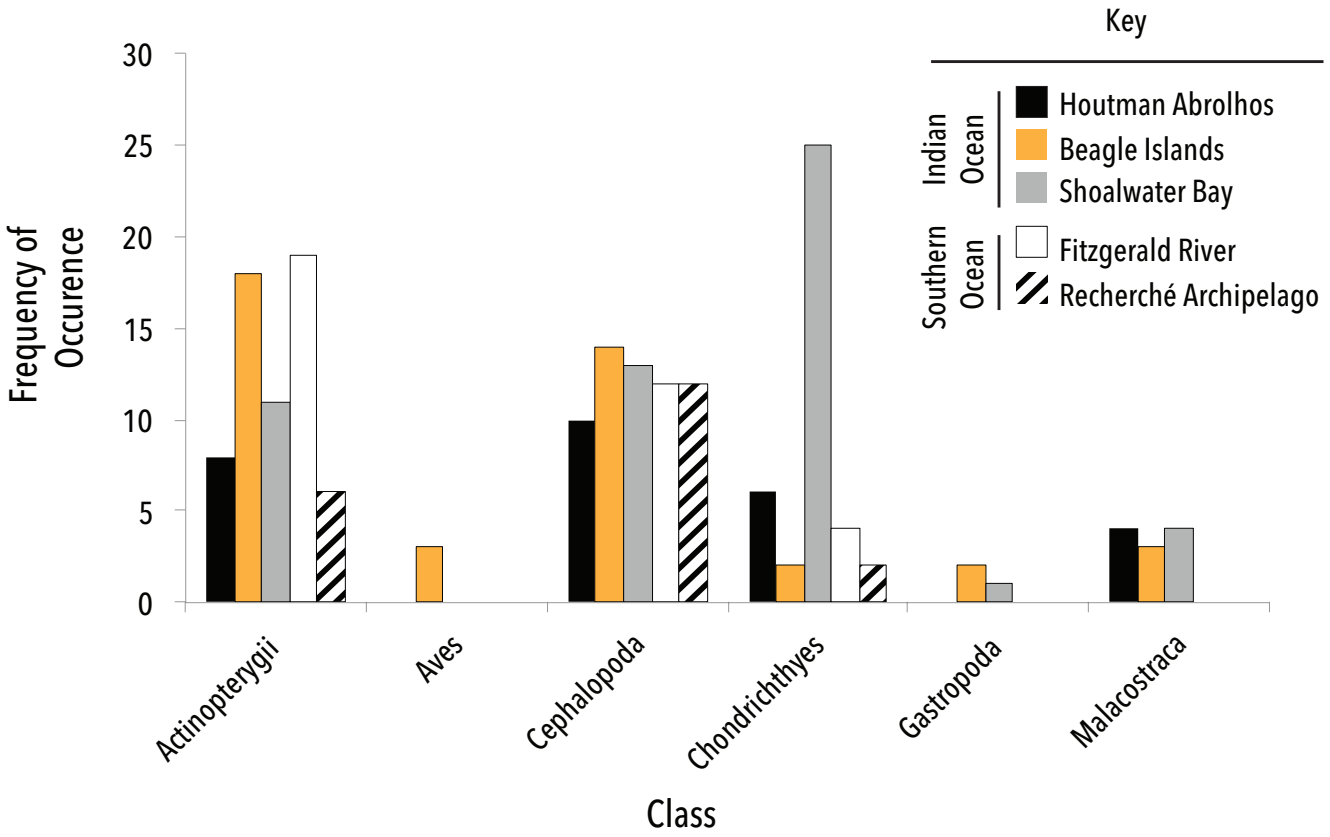

FIGURE 2.3 Sea lion diet: Classes of Taxa detected across the five WA study sites. The frequency a class of prey taxa was identified at each site using metabarcoding.

Overall the multi-gene metabarcoding generated in excess of 1.2 million NGS reads, which were converted to presence absence data. These assays revealed (Figure 2.3) that while the majority of the sea lion samples (~68%) contained both ray-finned fishes (Actinopterygii) and cephalopods (Cephalopoda), many sharks and rays (Chondrichthyes; ~22%) were also detected. This is especially true for those samples from Shoalwater Bay where Chondrichthyes made up the largest proportion of prey (~46%). The least common taxa were Aves and Gastropoda with only three detections each across the five sites. Table A2.3 shows the number of samples from each site that responded to each assay.

These findings are broadly consistent with the literature, although Kirkwood and Goldsworthy (2013) identify cephalopods as the top four sea lion prey items, followed by sharks and rays, lobsters and finally four species of ray-finned fishes. However, their study concentrates on sea lions from South Australian waters where species composition will differ to those in the WA sites studied here. The Indian Ocean sites also contained 11 incidences of malacostracans (a class of crustaceans that includes crayfish and shrimp) and three of gastropods (a class of molluscs which contains bivalves), whereas these taxa were absent from the Southern Ocean sites.

The majority of the identified prey are benthic, and are usually found at depths ≤ 150 m and most are found at < 80 m. This finding concurs with studies that suggest the maximum foraging diving depth for an adult male sea lion is 150 m (Kirkwood and Goldsworthy, 2013).

2.5.2 Sea lion diet – Fish detections

Fish sequences were detected using both the Fish 16S and the Plank COI assays. Together, the two metabarcoding assays identified 47 Actinopterygii—36 of which were assigned to a genus or species level—and 17 Chondrichthyes—13 of which were ascribed to a genus or species level (Tables A2.5 and A2.6).

While there was some redundancy in the two assays that target fish, typically they detected different taxa; only five of the taxa were detected by both assays (Table A2.5). The Fish 16S assay detected 72% of the ray-finned fishes compared with the Plank COI assay, which detected 38% of the ray-finned fishes identified. For the cartilaginous fish, this trend was reversed, with the Fish 16S assay detecting 41% of the taxa identified and the Plank COI assay yielding 71%; only one genus (*Mustelus*) was detected by both assays (Table A2.6). These results demonstrate that, even with broad-spectrum ("universal") PCR assays, important species are still missed, and that when metabarcoding assays are used in combination, they yield far more information about the biodiversity of environmental samples. This is because the biotic "background" will vary between sites/samples and "generic" primers will exhibit sample dependent bias, where, due to primer binding variation, one group of taxa will preferentially amplify over another where they are both present in the sample (Pompanon et al., 2012). These biases are manifest further when samples are in low copy number and/or inhibited (Murray et al., 2015).

Comparing sites, Perciformes were detected in all five samples from Houtman Abrolhos and the Beagle Islands, but were only detected in four of the six samples from Fitzgerald River, and were detected even less frequently in samples from Shoalwater Bay and Recherche Archipelago (Figure 2.4a). The order Perciformes contains a large variety of perch-like-fish including wrasse, parrotfish, goatfish and damselfish. Fifteen taxa from this order were detected overall, with the vast majority of these identified from the Beagle Islands samples. The likely reason for this is that while Perciformes are found in all areas of southern Western Australia, the majority of those species detected in the sea lion scat are mainly found in the Indian Ocean. An example of this is *Pomacanthus semicirculatus,* which has only been recorded in northern waters of Australia (ALA, 2016). There is also a climatic shift from the Indian (warmer) and Southern Oceans (cooler) that may result in differences in prey species for sea lions. Tetraodontiformes, which includes the family Monacanthidae (leatherjackets), also seems to be favoured across three sites (Beagle Island, Shoalwater Bay, and Fitzgerald River; Figure 2.4a). All these findings are in line with those of Peters et al. (2014), who also identified wrasse, goatfish and leatherjackets as important prey for sea lions.

Of note is the detection of eels (Anguilliformes) as prey, by both the Fish and Plank COI assays. The species detected include the highfin moray (*Gymnothorax pseudothyrsoideus*), conger eels (*Conger* and *Gnathophis*) and unknown species of knot eels (in the Muraenidae family). The consumption of eels by the sea lions has not previously been reported, and yet the frequency of occurrence (eight samples across all five sites) suggests this is a regular component of sea lion diet.

In contrast to the other sites, a large proportion of sharks and rays are consumed by sea lions at Shoalwater Bay (Figure 2.4b). Each of the ten samples from Shoalwater contained prey from all five orders of Chondrochthyes detected, including stingarees (Urolophidae) and wobbegongs (Orectolobidae). Even in the Houtman Abrolhos Islands, four out of the five samples produced sequences matching wobbegongs (Orectolobidae). While the Australian sea lion is known to eat sharks and rays (Kirkwood and Goldsworthy, 2013, Ling, 1992), it is suggested that many of the taxa identified here are previously unrecognized as sea lion prey.

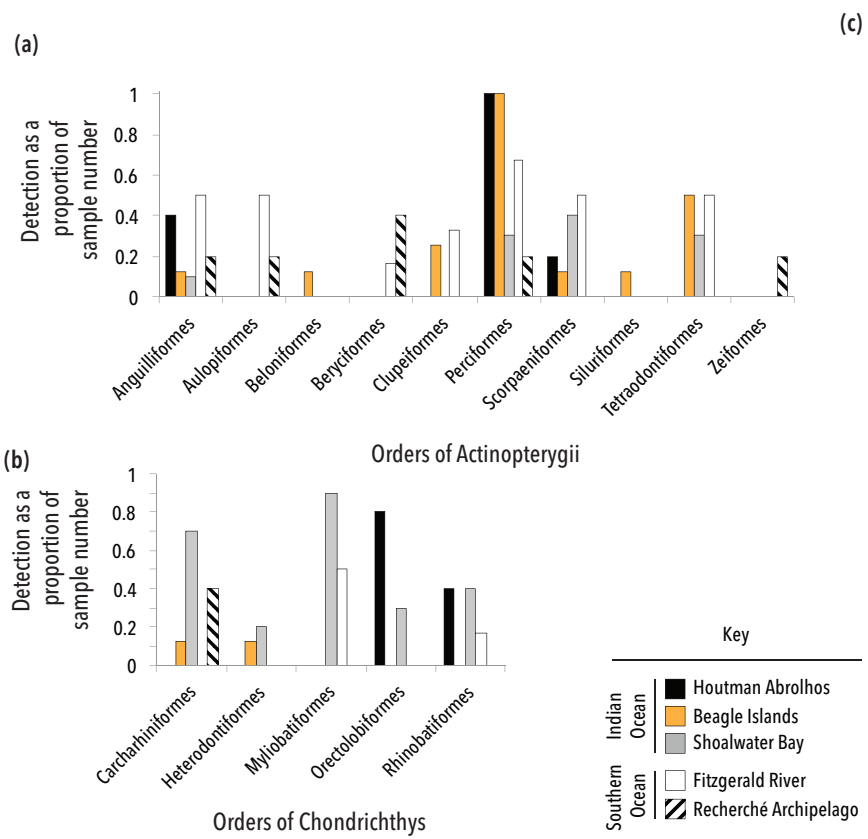

FIGURE 2.4 Metabarcoding of seal lion diet analyzed using ordinal and OTU assignments. The number of times an order within (a) Actinopterygii and (b) Chondrichthys were detected at each site as a proportion of the number of scat samples taken from each sample location. The OTU analysis of the Fish16S assay (c) demonstrates clear divisions between the genetic diversity of fish in the sample sites and between those samples sourced in the Indian Ocean compared with those from the Southern Ocean. The data used for (c) can be found in Table A2.9.

The vast majority of the fishes detected in this study are classified as demersal or benthic, and are found associated with reefs, seagrass, and the rocky and sandy bottoms of the continental shelf. This finding is consistent with current knowledge that describes the Australian sea lion as diving for its prey and being a principally benthic feeder (Kirkwood and Goldsworthy, 2013, Hesp et al., 2012, Gales and Cheal, 1992).

2.5.3 Sea Lion diet - Fish OTUs

There is a growing trend to move to taxonomic independent methods such as OTUs when describing genetic diversity in marine environments using metabarcoding data. This type of analysis allows for examination of all the available genetic diversity in metabarcoding data without the constraints of a frequently imprecise (and constantly evolving) taxonomic framework, coupled with an often-incomplete collection of reference DNA barcodes.

Given the large number of fish taxa detected (Tables A2.5 and A2.6) using the Fish 16S metabarcoding assay, we analyzed the estimated genetic diversity of fish between sites using OTUs. After filtering, a total of 38 OTUs (Table A2.9; at 97% clustering) were identified from the 34 samples across the five study sites. Clear differences in regionality of fish diversity among sites was observed, with only seven of the 38 (~18%) OTUs shared across two or more sites (Figure 2.4c). When these sites were grouped by ocean (i.e., Southern or Indian Ocean), the division in genetic diversity was even more obvious, with only three out of 38 (~8%) OTUs shared across the two oceans (Figure 2.4c).

From autumn to early spring (April to October) the Leeuwin Current (LC) brings warmer waters than would usually be found at these latitudes to the west coast of southern Western Australia, (as well as tropical fish and invertebrates; (Pearce and Feng, 2013)), with the result that water temperatures are maintained at a warmer level during winter. While this current continues around to the southern coastline, it is supplemented by currents from subantarctic waters (Cresswell and Domingues, 2009),

resulting in cooler environments in the Southern Ocean. Thus the clear genetic distinction in the Fish OTUs between the oceans is likely attributable to these differences in the habitats; although we cannot rule out that temporal differences have also contributed.

2.5.4 Sea lion diet - cephalopods & gastropods

Invertebrates, especially octopus, squid and cuttlefish are thought to make up a large proportion of the diet of the sea lions (McIntosh et al., 2006, Hesp et al., 2012, Peters et al., 2014, Kirkwood and Goldsworthy, 2013), but the actual invertebrate prey species remain largely unknown. The Plank COI, S_Ceph 16S and Ceph 16S metabarcoding assays identified 14 invertebrate taxa, with 11 identified to a genus or species level (Table A2.7). However, many of the octopus species nominally identified have not previously been described in the collection area (those not known in Australia were assigned to higher taxa). This may be because the S_Ceph primer set target is a small amplicon (~70 bp), and therefore one erroneous base, coupled with possible low interspecific variation at this locus, could result in erroneous assignments. The other possible reason is the relatively poor representation of the class on Genbank (of the taxa searched for in this study less than 75% had a 16S mtDNA sequence deposited in the database). However, as reference databases improve at widely utilized metabarcoding targets, so will our ability to make more robust taxonomic identifications.

Interestingly, the Plank COI assay also detected some cephalopods that provided additional support for taxa detected by both the Ceph and S_Ceph primers, in particular *Octopus* and *Sepia apama*. These two taxa were detected in 21 and 25 samples respectively, and across all sites (Figure 2.5).

Of particular interest is the detection of the southern calamari squid (*Sepioteuthis australis,* order Teuthida), an important commercial species in Australia. While this species was detected in samples across four of the five sites (Figure 2.5), it was detected in less than a quarter of all samples (~18%) and in these samples, this was not the only

prey revealed. This may indicate that the sea lions prefer octopus and giant cuttlefish to calamari, or it may suggest that the southern calamari squid is less abundant in the areas sampled. This latter possibility is perhaps more likely, as the occurrence records from the Atlas of Living Australia (2016) shows a decrease in the incidence of squid sightings west of the border with South Australia. Furthermore, in a South Australian sea lion study, Peters et al. (2014) also documented that *S. australis* is common prey.

The number of gastropod species detected was limited (Table A2.7) and these taxa have, to our knowledge, not been identified previously as potential sea lion prey. *Haliotis diversicolor* (many-colored abalone) is found in the area where it was detected (ALA, 2016) and while *Stomatella impertusa* (False ear shell) was represented by only a few sequences in one sample, it does reside in Australian waters and the Genbank record had a 100% match with the queried sequence. Despite observing these taxa in more than one scat, it is difficult to exclude the possibility that the observations may be a consequence of secondary predation (the carryover of DNA from the gut of ingested prey species).

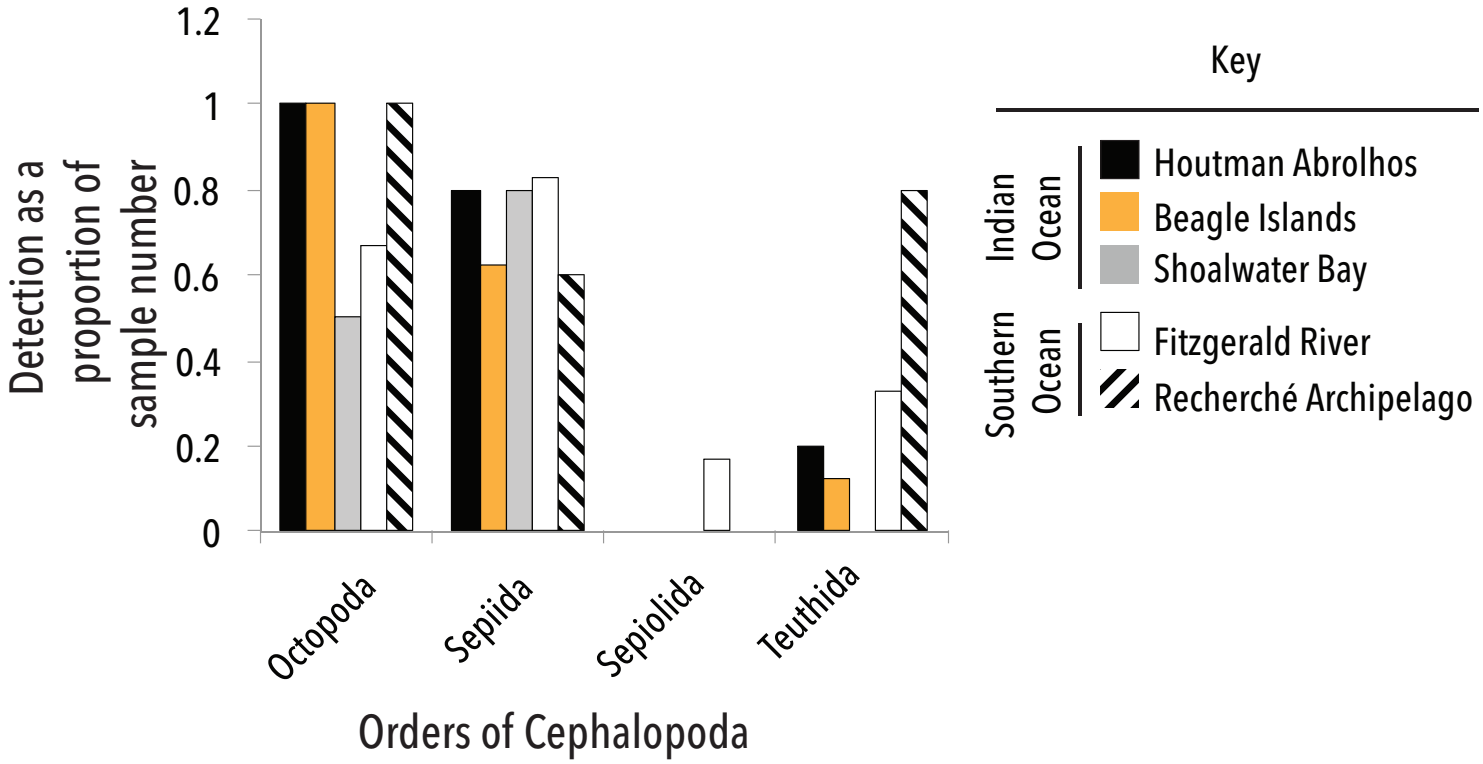

FIGURE 2.5 Sea lion diet: Orders of Cephalopod detected. The number of times an order within Cephalopoda was detected at each site as a proportion of the scat samples taken from each area. Data was obtained using the Ceph 16S, S_Ceph 16S and Plank COI assays.

2.5.5 Sea lion diet – crustaceans & birds

Crustaceans, including rock lobsters and swimming crabs, are noted as common prey of the Australian sea lion in South Australia (Kirkwood and Goldsworthy, 2013, McIntosh et al., 2006). The newly developed Crust16S assay detected five taxa, three to species level (Table A2.8). The results confirm that the Australian sea lion does prey on the commercially important western rock lobster (*Panulirus cygnus*), which was detected in six samples across all three sites in the Indian Ocean. This assay also detected a species of swimming crab (*Thalamita danae*) in a sample from the Houtman Abrolhos Islands; the only site where it is likely to be found (ALA, 2016).

The site at Shoalwater Bay is close to Penguin Island, which is home to a colony of little penguins (*Eudyptula minor*), a bird that is reported to be preyed upon by sea lions (McIntosh et al., 2006); as such, all samples were screened using the Bird 12S assay (which has been confirmed to detect *E. minor in silico* and *in vivo*), but no penguins were detected. However, we did detect the presence of one bird, a pied cormorant (*Phalocrocorax varius*), in three samples from Beagle Islands, which was also confirmed using the Plank COI assay. One of these samples also contained DNA from a bridled tern (*Onychoprion anaethetus*). While environmental contamination (e.g. sand on the beach, which was excluded as far as practicable) cannot be ruled out to explain the presence of both of these birds, neither can predation. Neither species of these birds has previously been documented as potential prey for Australian sea lions, but both are known to sit on the surface of the water (the pied cormorant also dives below the surface) and are thus susceptible to ambush predation from below.

Neither birds nor crustaceans were detected in the scats taken from the Southern Ocean sites. This may be because many of the crustaceans detected in the Indian Ocean are not known in the Southern Ocean and while there are decapods in the Southern Ocean they are not as prevalent as in other areas of Australia (ALA, 2016). However, since neither birds nor crustaceans appeared to make up a large proportion of the diet of the Indian Ocean sea lions, their absence in the diet of the Southern Ocean sea lions may be attributed merely to limited sample numbers, or prey preference at the time of sampling.

2.5.6 Spatial differences in sea lion diet

The nested PERMANOVA (adonis) analysis showed that taxa preyed upon by sea lions was significantly different among Sites ($p < .01$) and between the Indian and Southern Ocean ($p < .0001$). A metaMDS plot (stress = 0.1595043) was used to visualize the differences in taxonomic assemblages among the five sampling sites and between the Indian and Southern Oceans (Figure 2.6). There was obvious clustering for the oceanic data, however the distinctions between the individual sites were not as clear.

To investigate this, and despite the modest sample size, further PERMANOVA (adonis) analyses were conducted to explore potential differences within each ocean. These identified an overall significant difference between the three Indian Ocean sites ($p < .007$) but no significant variance among the two Southern Ocean sites. Subsequently, a pairwise adonis was used investigate which Indian Ocean sites were different; this revealed that the only significant difference was between Houtman Abrolhos and Beagle Islands ($p < .05$).

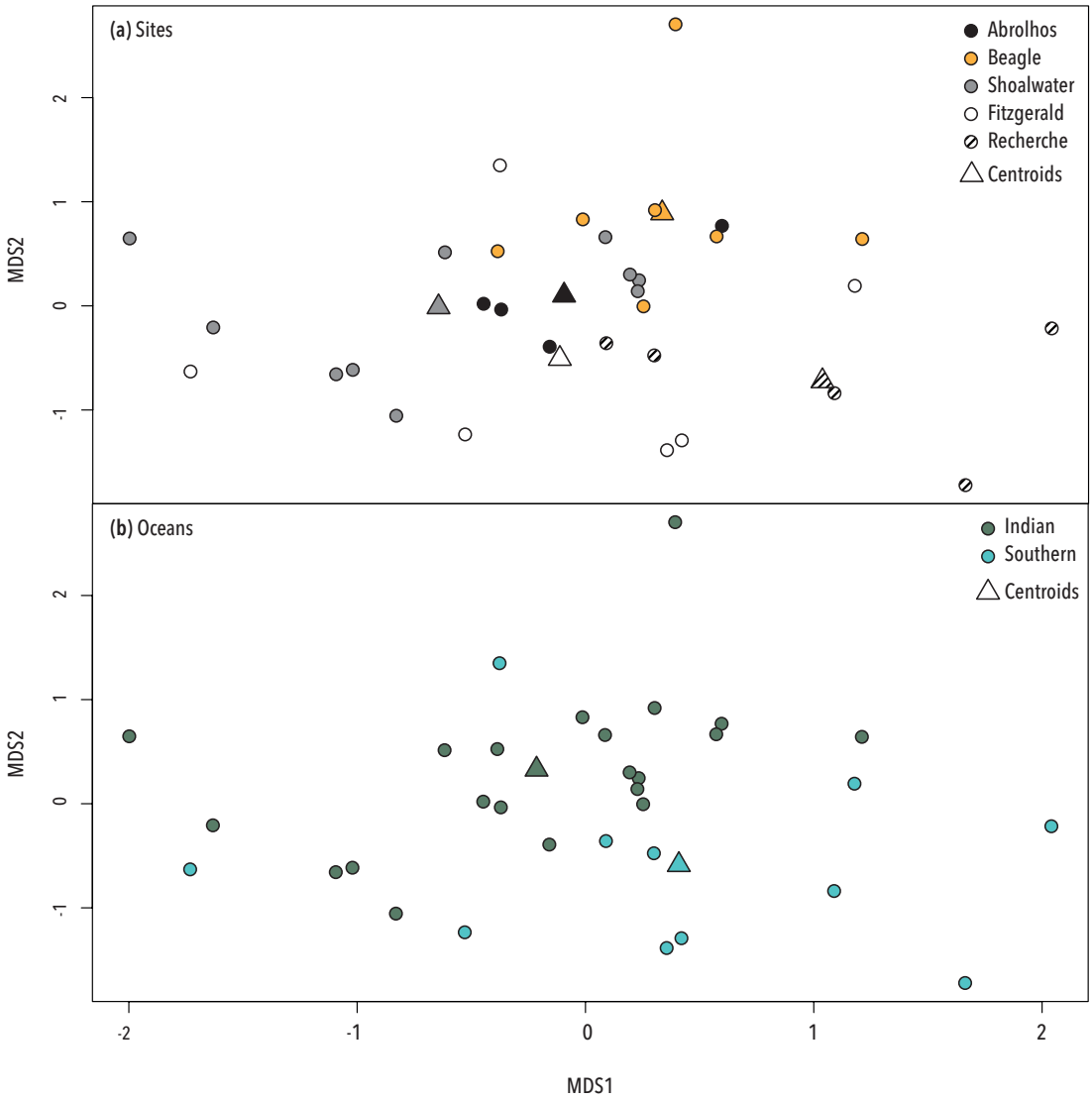

FIGURE 2.6 Multivariate analysis of all metabarcoding data assigned a taxonomic rank. (a) metaMDS plot comparing A taxa from the different sites of collection, and (b) the dietary differences between the sea lions of the Southern and Indian Oceans, centroids are marked with triangles.

To determine which taxa contributed to the significant differences in the PERMANOVA, indicator values analysis (indval) was performed. An indval analysis enables the taxa responsible for the regionality in the data to be discerned. While the 34 scats analyzed here are somewhat underpowered, the analysis is valuable due to the identification of taxa that drive the spatial patterns in the data. The indval analysis executed on the total metabarcoding dataset identified nine primary taxa that drive the variation in sea lion diet among sites ($p = .005 – .04$), and three primary taxa that drive the differences in taxonomic assemblage observed between the Indian and Southern Ocean ($p = .002 – .04$; Figure 2.7).

Given that birds and crustacea were only detected in the Indian Ocean it may have been expected that these taxa would drive differences between the two oceans. However, this is not the case; in the Indian Ocean it is *Octopus tetricus* that is flagged as a key indicator species and in the Southern Ocean it is fish; Aulopidae (threadsails) and in particular *Aulopus purpurissatus* (sergeant baker).

In the site indval analysis, Beagle Islands had four of the nine key indicator species (a bird (*P. varius*), some Actinopterygii (Monacanthidae and *Siganus*) and a species of *Octopus*. This is in keeping with the adonis analyses above, which showed Beagle Islands were significantly different from each of the other sites. Indicator taxa characterizing Shoalwater are predominantly carpet sharks, Orectolobidae and *Aulohalaelurus labiosus*. Carpet sharks (Orectolobidae and *Orectolobus*) are also the key indicator species for the Houtman Abrolhos Islands.

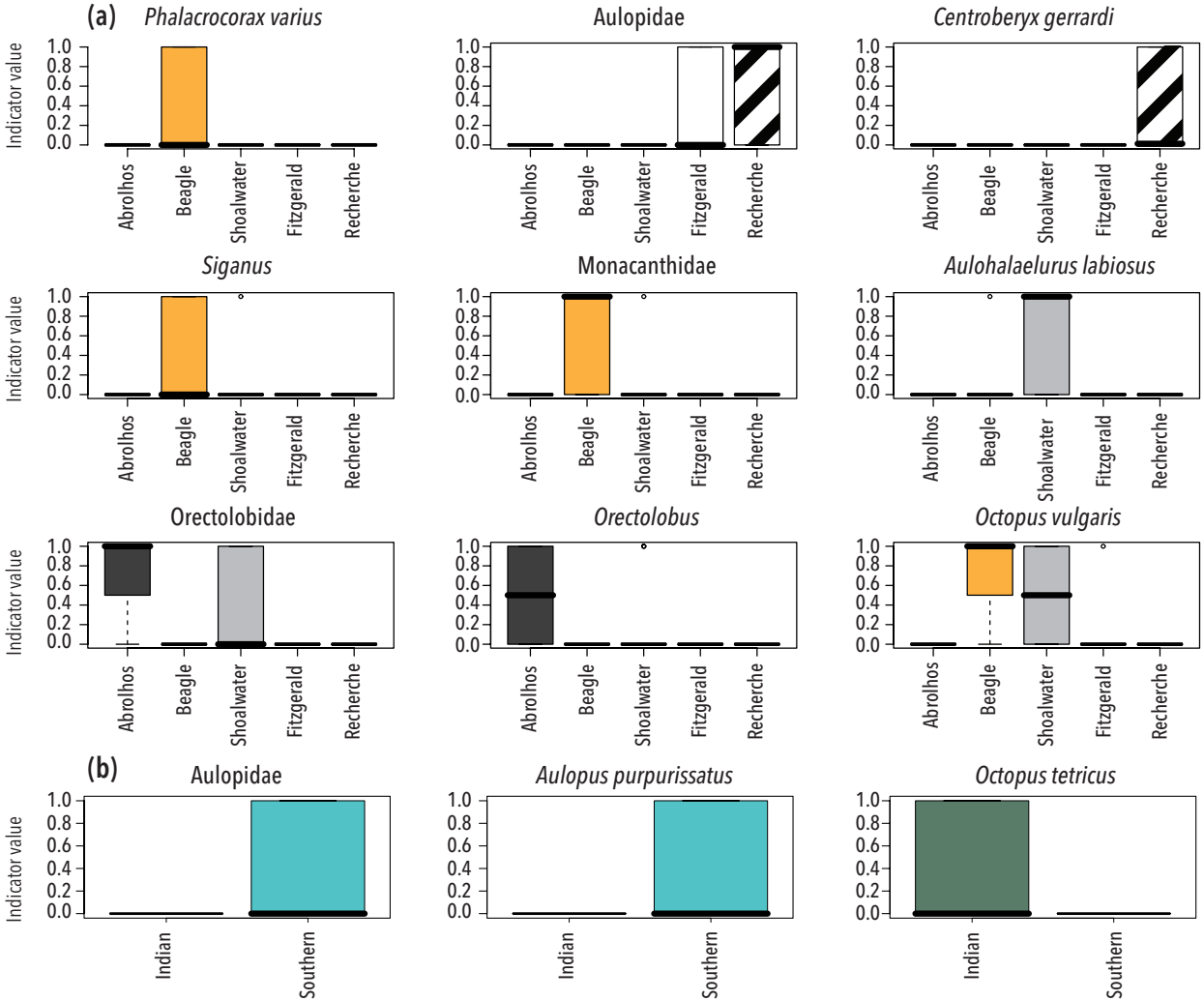

FIGURE 2.7 Indicator species analysis. Indval results from the total metabarcoding dataset showing the taxa characterizing each area and thus driving variations in sea lion diet between (a) sites, (b) oceans (all p values < .05).

Actinopterygii are the key indicator species in the Southern Ocean sites. Aulopidae are notable taxa at both Recherche and Fitzgerald, which is unsurprising as it was flagged as key indicator for differences found between the two oceans (Figure 2.7). In Recherche Archipelago *Centroberyx gerrardi* (red snapper), a commercial species, was also identified as an indicator species; although it was only found in two of the five samples taken from the area.

2.6 Conclusion

THIS was the first attempt to investigate and describe the diet of the Australian sea lion by DNA metabarcoding of scats. Despite the relatively small number of scats analyzed here (n=34) the results demonstrate the sensitivity of the approach to identify previously unrecorded species such as eels, gastropods and the frequency of sharks and rays in the diet. Importantly, metabarcoding offers a different method allowing identification taxa that are either difficult to detect through morphological analysis of feces or through direct observational studies. This study, like previous dietary studies using metabarcoding, have been somewhat hampered by lack of reference barcodes, but despite this limitation, the dietary audit presented here presents significant insight into the prey of this apex predator. Significantly, the comprehensiveness of these datasets will improve with time, and environmental data, such as generated here, can be re-analyzed. Finally, the data gathered from the scat of this endangered apex predator demonstrates that DNA metabarcoding is a relatively simple and non-invasive way to both monitor the sea lions' diet and to provide valuable insights into the regional biodiversity of our oceans. It is foreseen that the expansion of this type of project both temporally and spatially can only add to the information gathered presented here.

Less than half of the marine species detected in this dietary study are classified as commercial species (ALA, 2016, Froese, 2016). While it is clear the sea lions are preying on some commercial species (such as the commercially important western rock lobster, *P. cygnus,* and southern calamari squid, *S. australis*), sea lions are taking a large variety of prey and no particular commercial species seems to dominate their diet. The diversity of taxa exploited by the Australian sea lion between oceans, sites and even between samples, supports the notion that Australian sea lions are opportunistic feeders. This bodes well for the survival of this protected species, as (providing its core habitats are preserved), it's mode of feeding makes it more likely to adapt its diet to changes in the surrounding biodiversity.

2.7 References

ALA. 2016. *Atlas of Living Australia website* [Online]. http://www.ala.org.au. [Accessed September 2016].

ALTSCHUL, S. F., GISH, W., MILLER, W., MYERS, E. W. & LIPMAN, D. J. 1990. Basic local alignment search tool. *Journal of Molecular Biology,* 215**,** 403-410.

BENSON, D. A., KARSCH-MIZRACHI, I., CLARK, K., LIPMAN, D. J., OSTELL, J. & SAYERS, E. W. 2012. GenBank. *Nucleic Acids Res,* 40**,** D48-53.

BERRY, O., BULMAN, C., BUNCE, M., COGHLAN, M., MURRAY, D. C. & WARD, R. D. 2015. Comparison of morphological and DNA metabarcoding analyses of diets in exploited marine fishes. *Marine Ecology Progress Series,* 540**,** 167-181.

BOYER, S., CRUICKSHANK, R. H. & WRATTEN, S. D. 2015. Faeces of generalist predators as 'biodiversity capsules': A new tool for biodiversity assessment in remote and inaccessible habitats. *Food Webs,* 3**,** 1-6.

BROWN, D. S., JARMAN, S. N. & SYMONDSON, W. O. C. 2012. Pyrosequencing of prey DNA in reptile faeces: analysis of earthworm consumption by slow worms. *Molecular Ecology Resources,* 12**,** 259-266.

BUNCE, M., OSKAM, C. & ALLENTOFT, M. 2012. Quantitative Real-Time PCR in aDNA research. *In:* SHAPIRO, B. & HOFREITER, M. (eds.) *Ancient DNA: Methods and Protocols, Methods in Molecular Biology.* Springer Science+Business Media.

CASPER, R. M., JARMAN, S. N., GALES, N. J. & HINDELL, M. A. 2007. Combining DNA and morphological analyses of faecal samples improves insight into trophic interactions: a case study using a generalist predator. *Marine Biology,* 152**,** 815-825.

COOPER, A. 1994. *DNA from museum specimens,* New York, Springer.

COSTA, D. P. & GALES, N. J. 2003. Energetics Of A Benthic Diver: Seasonal Foraging Ecology Of The Australian Sea Lion, *Neophoca Cinerea*. *Ecological monographs,* 73**,** 27-43.

CRESSWELL, G. & DOMINGUES, C. M. 2009. Leeuwin Current A2 - Steele, John H. *Encyclopedia of Ocean Sciences (Second Edition).* Oxford: Academic Press.

DEAGLE, B. E., GALES, N. J., EVANS, K., JARMAN, S. N., ROBINSON, S., TREBILCO, R. & HINDELL, M. A. 2007. Studying Seabird Diet through Genetic Analysis of Faeces: A Case Study on Macaroni Penguins (*Eudyptes chrysolophus*). *PLoS ONE,* 2**,** e831.

DEAGLE, B. E., KIRKWOOD, R. & JARMAN, S. N. 2009. Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces. *Molecular Ecology,* 18**,** 2022-2038.

DEAGLE, B. E., TOLLIT, D. J., JARMAN, S. N., HINDELL, M. A., TRITES, A. W. & GALES, N. J. 2005. Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions. *Mol Ecol,* 14**,** 1831-42.

DELPORT, T. C., POWER, M. L., HARCOURT, R. G., WEBSTER, K. N. & TETU, S. G. 2016. Colony Location and Captivity Influence the Gut Microbial Community Composition of the Australian Sea Lion (*Neophoca cinerea*). *Appl Environ Microbiol,* 82**,** 3440-9.

EDGAR, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics,* 26**,** 2460-2461 %@ 1367-4803.

FROESE, R. A. P., D. 2016. *Fishbase* [Online]. http://www.fishbase.org: World Wide Web electronic publication. [Accessed 2016].

GALES, N. J. & CHEAL, A. J. 1992. Estimating Diet Composition Of The Australian Sea-Lion (*Neophoca Cinerea*) From Scat Analysis - An Unreliable Technique. *Wildlife Research,* 19**,** 447-456.

GOLDSWORTHY, S., MCKENZIE, J., SHAUGHNESSY, P., MCINTOSH, R., PAGE, B. & CAMPBELL, R. 2009. An update of the report: understanding the impediments to the growth of Australian sea lion populations. *SARDI research report series*.

GOLDSWORTHY, S. D. 2015. *Neophoca cinerea, Australian Sea Lion. The IUCN Red List of Threatened Species.* http://dx.doi.org/10.2305/IUCN.UK.2015-2.RLTS.T14549A45228341.en.

HARDY, N., BERRY, T., KELAHER, B. P., GOLDSWORTHY, S. D., BUNCE, M., COLEMAN, M. A., GILLANDERS, B. M., CONNELL, S. D., BLEWITT, M. & FIGUEIRA, W. 2017. Assessing the trophic ecology of top predators across a recolonisation frontier using DNA metabarcoding of diets. *Marine Ecology Progress Series,* 573**,** 237-254.

HESP, S. A., TWEEDLEY, J. R., MCAULEY, R., TINK, C. J., CAMPBELL, R. A., CHUWEN, B. M. & HALL, N. G. 2012. Informing tisk assessment through estimating interaction rates between Australian sea lions and Western Austrlaia's temperate demersal gillnet fisheries. *Fisheries Research and Development Corporation Report.* Murdoch: Centre for Fish and Fisheries Research, Murdoch University.

HIBERT, F., TABERLET, P., CHAVE, J., SCOTTI-SAINTAGNE, C., SABATIER, D. & RICHARD-HANSEN, C. 2013. Unveiling the diet of elusive rainforest herbivores in next generation sequencing era? The tapir as a case study. *PloS one,* 8.

HUSON, D. H., MITRA, S., RUSCHEWEYH, H. J., WEBER, N. & SCHUSTER, S. C. 2011. Integrative analysis of environmental sequences using MEGAN 4, . *Genome Res.,* 21**,** 1552-1560.

KEARSE, M., MOIR, R., WILSON, A., STONES-HAVAS, S., CHEUNG, M., STURROCK, S., BUXTON, S., COOPER, A., MARKOWITZ, S., DURAN, C., THIERER, T., ASHTON, B., MEINTJES, P. & DRUMMOND, A. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics,* 28**,** 1647-1649.

KIRKWOOD, R. & GOLDSWORTHY, S. 2013. *Fur Seals and Sea Lions,* Collingwood, Victoria, CSIRO Publishing.

LING, J. K. 1992. Neophoca cinerea. *Mammalian Species,* 392**,** 1-7.

MCINTOSH, R. R., PAGE, B. & GOLDSWORTHY, S. D. 2006. Dietary analysis of regurgitates and stomach samples from free-living Australian sea lions. *Wildlife Research,* 33**,** 661-669.

MCLAUGHLIN, M. J. & SAINANI, K. L. 2014. Bonferroni, Holm, and Hochberg corrections: fun names, serious changes to p values. *PM R,* 6**,** 544-6.

MURRAY, D. C., BUNCE, M., CANNELL, B. L., OLIVER, R., HOUSTON, J., WHITE, N. E., BARRERO, R. A., BELLGARD, M. I. & HAILE, J. 2011. DNA-Based Faecal Dietary Analysis: A Comparison of qPCR and High Throughput Sequencing Approaches. *Plos One,* 6.

MURRAY, D. C., COGHLAN, M. L. & BUNCE, M. 2015. From benchtop to desktop: important considerations when designing amplicon sequencing workflows. *PloS one,* 10**,** e0124671.

OKSANEN, J., GUILLAUME BLANCHET, F., FRIENDLY, M., KINDT, R., LEGENDRE, P., MCGLINN, D., MINCHIN, P. R., O'HARA, R. B., SIMPSON, G. L., SOLYMOS, P., STEVENS, M. H. H., SZOECS, E. & WAGNER, H. 2016. vegan: Community Ecology Package. R package version 2.3-0. http://CRAN.R-project.org/package=vegan.

OSTERRIEDER, S. K., SALGADO KENT, C. & ROBINSON, R. W. 2015. Variability in haul-out behaviour by male Australian sea lions Neophoca cinerea in the Perth metropolitan area, Western Australia. *Endangered Species Research,* 28**,** 259-274.

OSTERRIEDER, S. K., SALGADO KENT, C. & ROBINSON, R. W. 2016. Responses of Australian sea lions, Neophoca cinerea, to anthropogenic activities in the Perth metropolitan area, Western Australia. *Aquatic Conservation: Marine and Freshwater Ecosystems*.

PEARCE, A. F. & FENG, M. 2013. The rise and fall of the "marine heat wave" off Western Australia during the summer of 2010/2011. *Journal of Marine Systems,* 111-112**,** 139-156.

PETERS, K. J., OPHELKELLER, K., BOTT, N. J., DEAGLE, B. E., JARMAN, S. N. & GOLDSWORTHY, S. D. 2014. Fine-scale diet of the Australian sea lion

(Neophoca cinerea) using DNA-based analysis of faeces. *Marine Ecology***,** n/a-n/a.

PETERS, K. J., OPHELKELLER, K., HERDINA, BOTT, N. J. & GOLDSWORTHY, S. D. 2015. PCR-based techniques to determine diet of the Australian sea lion (*Neophoca cinerea*): a comparison with morphological analysis. *Marine Ecology,* 36**,** 1428-1439.

POMPANON, F., DEAGLE, B. E., SYMONDSON, W. O., BROWN, D. S., JARMAN, S. N. & TABERLET, P. 2012. Who is eating what: diet assessment using next generation sequencing. *Mol Ecol,* 21**,** 1931-50.

QUEMERE, E., HIBERT, F., MIQUEL, C., LHUILLIER, E., RASOLONDRAIBE, E., CHAMPEAU, J., RABARIVOLA, C., NUSBAUMER, L., CHATELAIN, C., GAUTIER, L., RANIRISON, P., CROUAU-ROY, B., TABERLET, P. & CHIKHI, L. 2013. A DNA Metabarcoding Study of a Primate Dietary Diversity and Plasticity across Its Entire Fragmented Range. *PloS one,* 8**,** e58971-e58971.

R CORE TEAM. 2015. *R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria* [Online]. Vienna, Austria. Available: http://www.R-project.org/. [Accessed 2016].

RADULOVICI, A. E., SAINTE-MARIE, B. & DUFRESNE, F. 2009. DNA barcoding of marine crustaceans from the Estuary and Gulf of St Lawrence: a regional-scale approach. *Molecular Ecology Resources,* 9**,** 181-187.

ROBERTS, D. W. 2016. labdsv: Ordination and Multivariate Analysis for Ecology. R package version 1.8-0.

SHEHZAD, W., MCCARTHY, T. M., POMPANON, F., PUREVJAV, L., COISSAC, E., RIAZ, T. & TABERLET, P. 2012a. Prey Preference of Snow Leopard (*Panthera uncia*) in South Gobi, Mongolia. *Plos One,* 7.

SHEHZAD, W., RIAZ, T., NAWAZ, M. A., MIQUEL, C., POILLOT, C., SHAH, S. A., POMPANON, F., COISSAC, E. & TABERLET, P. 2012b. Carnivore diet analysis based on next-generation sequencing: application to the leopard cat (*Prionailurus bengalensis*) in Pakistan. *Molecular Ecology,* 21**,** 1951-1965.

TAYLOR, P. G. 1996. Reproducibility of Ancient DNA Sequences from Extinct Pleistocene Fauna. *Molecular Biology and Evolution,* 13**,** 283-285.

THOMAS, A. C., JARMAN, S. N., HAMAN, K. H., TRITES, A. W. & DEAGLE, B. E. 2014. Improving accuracy of DNA diet estimates using food tissue control materials and an evaluation of proxies for digestion bias. *Mol Ecol,* 23**,** 3706-18.

2.8 Appendix

TABLE A2.1 Sample collection data; details of collection dates and sites and number of scats collected

Reserve	Ocean	No. Samples	Date collected
Houtman Abrolhos Nature Reserve	Indian	5	10 April 2013
Beagle Islands Nature Reserve	Indian	8	17 May 2013
Shoalwater Islands Nature Reserve	Indian	1 5 4	October 2012 20/21 January 2013 22 May 2013
Fitzgerald River Nature Reserve	Southern	6	October 2012
Recherche Archipelago Nature Reserve	Southern	4 1	26 January 2013 27 January 2013

Class	Assignment	Metabarcoding assay and % match of query to reference			
		Crust (170 bp)	Fish (200 bp)	S_Ceph (70 bp)	Ceph (200 bp)
Actinopterygii	Encrasicholina punctifer		99-100		
	Hyporhamphus melanochir		99-100		
	Sardinops (sagax/neopilchardus)		99-100		
	Spratelloides robustus		99-100		
Malacostraca	Fenneropenaeus merguiensis	99-100			
	Portunus pelagicus	99-100			
Cephalopoda	Nototodarus sloanii				98-100
	Ommastrephidae (Martialia hyadesi /Nototodarus sloanii /Todarodes filippovae)			97-100	

Table A2.2: Single source analysis of metabarcoding assays; details of assays tested against DNA extracted from single source samples (barcode size in brackets).

Assay	Houtman Abrolhos (6)	Beagle (8)	Shoalwater Bay (10)	Fitzgerald River (6)	Recherche Archipelago (5)
	Number of samples producing results				
Bird 12S	0	3	0	0	0
Ceph 16S	4	6	9	4	1
Crust 16S	3	3	5	0	0
Fish 16S	4	5	8	4	3
Mam 16S	6	8	10	6	5
Plank COI	5	8	10	5	4
S_Ceph 16S	4	6	9	6	5

TABLE A2.3 Number of samples producing results for each assay; the total number of samples from each site are in brackets.

TABLE A2.4 Numbers of sequences per assay, per site; 'Unfiltered' refers to sequences that have been 100% matched to the sequence specific primers, the MID tags and the adaptor sequence. 'Filtered and assigned' refers to the number of sequences that have passed through the Usearch filtering process and were assigned to taxa. 'Mean unique' refers to the mean number of unique sequences produced given the number of positive samples for the assay.

Site	Sequence type	Ceph 16S	S_Ceph 16S	Fish 16S	Plank COI	Crust 16S
Houtman Abrolhos	Unfiltered	36225	114540	56420	30530	34167
	Mean unique	395 ± 431	694 ± 453	1487 ± 367	324 ± 138	622 ± 504
	Filtered and assigned	33164	108929	42584	28499	30999
	Mean unique	55 ± 16	95 ± 13	34 ± 25	65 ± 25	34 ± 12
Beagle Islands	Unfiltered	23183	147703	73954	42126	30913
	Mean unique	327 ± 403	555 ± 208	1685 ± 479	309 ± 126	898 ± 518
	Filtered and assigned	19909	140073	53570	38603	25559
	Mean unique	35 ± 14	88 ± 23	25 ± 4	77 ± 32	47 ± 21
Shoalwater Bay	Unfiltered	34613	146541	92175	52095	81945
	Mean unique	331 ± 250	479 ± 331	1385 ± 745	259 ± 148	862 ± 266
	Filtered and assigned	29110	125647	50835	41130	41899
	Mean unique	46 ± 19	81 ± 22	26 ± 18	43 ± 18	51 ± 8
Fitzgerald River	Unfiltered	7754	50363	94898	15122	0
	Mean unique	168 ± 125	368 ± 143	2301 ± 742	198 ± 113	0
	Filtered and assigned	3624	45758	57549	14045	0
	Mean unique	59 ± 47	74 ± 11	37 ± 25	60 ± 40	0
Recherche Archipelago	Unfiltered	332	55926	36321	20501	0
	Mean unique	50	534 ± 124	1239 ± 165	218 ± 160	0
	Filtered and assigned	325	46432	27500	19472	0
	Mean unique	48	62 ± 31	29 ± 13	62 ± 42	0

TABLE A2.5 Actinopterygii (ray-finned fishes) identified with Plank COI and Fish 16S assays. The number of samples in which the taxa were detected is indicated in the brackets

Order	Family	Genus/Species	Common name	Abrolhos	Beagle	Shoalwater	Fitzgerald	Recherche
Anguilliformes	Congridae	*Conger*	Genus of Conger eels		Plank (1)	Plank (1)	Plank (2), Fish (2)	
		Gnathophis	Genus of Conger eels				Fish (1)	
	Muraenidae		Knot-eels	Fish (1)				Fish (1)
		Gymnothorax pseudothyrsoideus	Highfin Moray	Fish (1)				
Aulopiformes	Aulopidae		Aulopus				Plank (2)	Plank (3)
		Aulopus purpurissatus	Sergeant Baker				Fish (3)	Fish (1)
Beloniformes	Exocoetidae		Flying fishes		Fish (1)			
Beryciformes	Anoplogastridae		Fangtooths				Fish (1)	
	Berycidae	*Centroberyx australis*	Yellow-eyed Red Snapper					Fish (1)
		Centroberyx gerrardi	Red Snapper					Plank (2)
Clupeiformes	Clupeidae		Herrings		Fish (1)			
		Etrumeus	Maray				Plank (1)	
		Sardinops sagax	Australian Sardine				Fish (1)	
	Engraulidae	*Engraulis*	Anchovies		Fish (1)			
Perciformes			Perch-like Fishes			Fish (1)		
	Labridae		Wrasses		Fish (1)			
		Coris auricularis	Western King Wrasse		Plank (1)			
		Leptoscarus vaigiensis	Marbled Parrotfish	Fish (1)				
		Notolabrus	Wrasses		Plank (2)			
		Odax acroptilus	Marine Rainbowfish		Fish (1)			
		Odax cyanomelas	Herring Cale				Plank(2), Fish (1)	
	Mullidae	*Parupeneus*	Common Goatfish	Fish (1)				
		Parupeneus spilurus	Blacksaddle Goatfish	Plank (1)				
		Upeneichthys	Goatfish		Fish (1)	Fish (1)	Fish (1)	
		Upeneichthys stotti	Stott's Goatfish		Fish (1)		Fish (1)	
	Pempheridae	*Parapriacanthus*	Bullseyes				Fish (1)	

Order	Family	Genus/Species	Common name	Abrolhos	Beagle	Shoalwater	Fitzgerald	Recherche
	Pomacanthidae	*Pomacanthus semicirculatus*	Blue Angelfish	Fish (1)				
	Pomacentridae		Damselfishes	Plank (2)	Plank (1)			
		Chromis	Damselfishes		Fish (1)			
		Parma microlepis	White Ear		Fish (1)			
	Siganidae	*Siganus*	Rabbitfish		Fish (2), Plank (2)	Fish (1)		
Scorpaeniformes			Scorpion Fishes & Sculpins				Fish (1)	
	Platycephalidae	*Leviprora inops*	Crocodile Flathead	Plank (2)	Plank (1)	Plank (4)		
		Platycephalus aurimaculatus	Tiger Flathead				Plank (2)	
		Platycephalus longispinis	Long-spine Flathead				Plank (1)	
	Scorpaenidae	*Scorpaenodes*	Scorpion Fish				Fish (1)	
Siluriformes	Plotosidae		Blunt-tail Catfishes		Plank (1)			
Tetraodontiformes	Monacanthidae		Leatherjackets		Fish (1)			
		Chaetodermis penicilligera	Tasselled Leatherjacket		Fish (1), Plank (1)	Fish (2), Plank (1)		
		Eubalichthys mosaicus	Mosaic Leatherjacket		Fish (1)			
		Monacanthus chinensis	Fanbelly Leatherjacket		Fish (1)			
		Nelusetta ayraudi	Ocean Jacket				Plank (1)	
		Scobinichthys granulatus	Rough Leatherjacket		Fish (1), Plank (2)			
		Thamnaconus	Leatherjacket			Fish (1)		
	Tetraodontidae	*Omegophora*	Toadfish				Fish (1)	
		Omegophora armilla	Ringed Toadfish				Plank (2)	
Zeiformes	Zeidae	*Zeus faber*	John Dory					Fish (1)

TABLE A2.6 Chondrichthyes (sharks and rays) identified with Plank COI and Fish 16S assays. The number of samples in which the taxa was detected is indicated in the brackets.

Order	Family	Genus/Species	Common name	Abrolhos	Beagle	Shoalwater	Fitzgerald	Recherche
Carcharhiniformes			Ground sharks			Fish (2)		Plank (1)
	Scyliorhinidae	*Aulohalaelurus labiosus*	Black Spotted Catshark		Plank (1)	Plank (6)		
	Triakidae	*Mustelus*	Gummy shark					Fish (1), Plank (1)
Heterodontiformes	Heterodontidae	*Heterodontus*				Fish (1)		
		Heterodontus portusjacksoni	Port Jackson Shark		Plank (1)	Plank (2)		
Myliobatiformes	Myliobatidae	*Myliobatis australis*	Southern Eagle Ray			Plank (1)		
	Urolophidae		Stingarees/Round Rays			Fish (1)		
		Trygonoptera				Plank (1)	Plank (1)	
		Trygonoptera mucosa	Western Shovelnosed Stingaree				Plank (1)	
		Trygonoptera personata	Masked Stingaree			Plank (3)	Plank (1)	
		Urolophus lobatus	Lobed Stingaree			Plank (3)	Plank (1)	
		Urolophus paucimaculatus	Sparsely Spotted Stigaree			Plank (1)	Plank (1)	
Orectolobiformes	Orectolobidae		Wobbegongs	Plank (4)		Plank (3)		
		Orectolobus		Fish (2)		Fish (2)		
Rhinobatiformes	*Rhinobatidae*		Guitarfishes	Fish (1)		Fish (2)		
		Aptychotrema vincentiana	Western Shovelnose Ray	Plank (1)		Plank (1)		
		Trygonorrhina guanerius	Southern Fiddler Ray			Fish (1)		

TABLE A2.7 Cephalopod and Gastropod taxa identified with Ceph 16S, S_Ceph 16S and Plank COI assays. The number of samples in which the taxa was detected is indicated in the brackets. Those species found in Australia, but not in the area of collection, are indicated by an asterisk (*).

Class	Order	Family	Genus/Species	Common name	Abrolhos	Beagle	Shoalwater	Fitzgerald	Recherche
Cephalopoda	Octopodiformes	Octopodidae		Octopus	S_Ceph (2)	S_Ceph (8)	S_Ceph (1)	S_Ceph (2) Ceph (1)	
			*Grimpella thaumastocheir**	Velvet Octopus	S_Ceph (1)	S_Ceph (2)			
			Octopus		S_Ceph (4), Plank (2), Ceph (1)	S_Ceph (6), Plank (3)	S_Ceph (5), Plank (4)	S_Ceph (2)	S_Ceph (2)
			*Octopus ornatus**		Ceph (1)	Ceph (1)			
			Octopus tetricus	Gloomy Octopus	S_Ceph (1)	S_Ceph (3)	S_Ceph (5)		
			*Octopus vulgaris**	Common Octopus		S_Ceph (3), Ceph (4)	S_Ceph (4), Ceph (5)	Ceph (1)	
			Scaeurgus					S_Ceph (1)	
	Oegopsida	Ommastrephidae	*Nototodarus gouldi/ Todarodes pacificus**						S_Ceph (2)
			Nototodarus gouldi	Red Arrow Squid					Ceph (1)
	Myopsida	Loliginidae	*Sepioteuthis australis*	Southern Calamari Squid	S_Ceph (1), Ceph (1)	S_Ceph (1), Ceph (1)		S_Ceph (2), Ceph (1)	S_Ceph (2)
	Sepiida	Sepiidae		Cuttlefish					S_Ceph (1), Plank(1)
			Sepia apama	Giant Cuttlefish	S_Ceph(4), Plank (4), Ceph (4)	S_Ceph (5), Plank (3), Ceph (5)	S_Ceph (8), Plank (5), Ceph (7)	S_Ceph (5), Plank (4), Ceph (1)	S_Ceph (2)
		Sepiolidae		Dumpling Squids				S_Ceph (1)	
Gastropoda		Haliotidae	*Haliotis*			S_Ceph (1)	S_Ceph (1), Ceph (1)		
			Haliotis diversicolor	Many Colored Abalone		S_Ceph (2)			
		Trochidae	*Stomatella impertusa*	False ear shell				S_Ceph (1)	

TABLE A2.8 Crustaceans and Aves detected using Crust 16S, Bird 12S and Plank COI assays. The number of samples in which the taxa was detected is indicated in the brackets.

Class	Order	Family	Genus/Species	Common name	Abrolhos	Beagle	Shoalwater	Fitzgerald	Recherche
Malacostraca	Decapoda			Crustaceans			Crust (1)		
		Palinuridae	*Panulirus cygnus*	Western Rock Lobster	Crust (1)	Crust (3)	Crust (2)		
		Portunoidea	*Thalamita danae*	Unknown Swimmer Crab	Crust (1)				
		Xanthidae		Crabes de Boue	Crust (1)				
Aves	Charadriformes	Laridae	*Onychoprion anaethetus*	Bridled Tern		Plank (1)			
	Suliformes	Phalacrocoracidae	*Phalacrocorax varius*	Pied Cormorant		Plank (2), Bird (3)			

TABLE A2.9 Fish 16S OTU sequence abundance per site and per ocean

OTU ID	Abrolhos	Beagle	Shoalwater	Fitzgerald	Recherche	Indian Ocean	Southern Ocean
OTU_1	13242	0	0	0	0	13242	0
OTU_7	7980	0	0	0	0	7980	0
OTU_16	8964	0	0	0	0	8964	0
OTU_29	1532	0	0	0	0	1532	0
OTU_30	1441	0	0	0	0	1441	0
OTU_34	1536	0	0	0	0	1536	0
OTU_38	267	0	0	0	0	267	0
OTU_6	0	8456	0	0	0	8456	0
OTU_12	0	7816	0	0	0	7816	0
OTU_19	0	4092	0	0	0	4092	0
OTU_21	0	3106	0	0	0	3106	0
OTU_27	0	2165	0	0	0	2165	0
OTU_28	0	1685	0	0	0	1685	0
OTU_31	0	1160	0	0	0	1160	0
OTU_35	0	851	0	0	0	851	0
OTU_8	0	11384	3620	0	0	15004	0
OTU_10	0	8972	10141	0	0	19113	0
OTU_14	0	0	5014	0	0	5014	0
OTU_15	0	0	7118	0	0	7118	0
OTU_17	0	0	4395	0	0	4395	0
OTU_20	0	0	3137	0	0	3137	0
OTU_22	0	0	2719	0	0	2719	0
OTU_23	0	0	5008	4	0	5008	4
OTU_25	0	0	2386	0	0	2386	0
OTU_11	4329	0	6858	96	0	11187	96
OTU_5	0	46	10736	10787	0	10782	10787
OTU_36	0	117	0	1659	0	117	1659
OTU_37	0	17	0	315	0	17	315
OTU_3	0	0	0	11830	0	0	11830
OTU_4	0	0	0	11695	0	0	11695
OTU_13	0	0	0	7989	0	0	7989
OTU_24	0	0	0	2414	0	0	2414
OTU_32	0	0	0	1913	0	0	1913
OTU_2	0	0	0	16951	10632	0	27583
OTU_9	0	0	0	0	7835	0	7835
OTU_18	0	0	0	0	4375	0	4375
OTU_26	0	0	0	0	2016	0	2016
OTU_33	0	0	0	0	1011	0	1011

2.9 Close

THIS paper captures the first published attempt to describe the diet of the endangered Australian sea lion using both next generation sequencing and multi-gene metabarcoding of eDNA. The study proved the methods as valuable techniques to both monitor the diet and to sample the biodiversity of the sea lion's habitat. Some hitherto unrecorded prey were identified and insight into the spatial and opportunistic nature of the sea lions' dietary choices was provided. It was determined that while the sea lions were targeting some commercial species, these did not form the majority of the sea lions' diet. In addition, the study provided three new assays with which to probe DNA from complex marine environmental samples.

The research provided the impetus for a collaborative project with the University of New South Wales. The collaboration involved design and implementation of the laboratory and bioinformatics stages of the work, as well as providing background information. The investigation involved characterising the diets of the Australian fur seal (*Arctocephalus pusillus doriferus*) and the long nosed fur seal (*A. forsteri*). Five of the most useful assays from the sea lion study were used in the collaboration, including two of the novel assays. The research resulted in a second author publication in the *Marine Ecology Progress Series* (Hardy et al (2017) Vol. 573, pg. 237-254); a copy of the abstract for this paper can be found in the Appendix.

While this pilot study added to the growing literature about the Australian sea lion, what it eats and it's habitats, it provides little information as to how this changes over time. The feasibility of the approach taken was well substantiated and, despite the small size of the study, statistically significant site differences were found. However, if a more temporal and complete picture is to emerge, much longer sampling periods are needed.

Here a multi-gene metabarcoding approach was modified and assessed for use with opportunistic samples from an apex predator. Yet the results obtained are limited to those taxa the sea lion chooses to predate. Life within the ocean is highly diverse and much of it is far too small to be of any interest to a self-respecting sea lion. Chapter 3 sees the further development of suitable methods to explore a different and far more complex environmental marine eDNA substrate.

INTERLUDE

An Ode to the Planktonic Relationships of Rottnest Island
- A five-year metabarcoding study

Adapted from a poster presentation for the Society for Molecular Biology and Evolution conference (Gold Coast), and Combined Biological Sciences Meeting (Perth) in 2016.

Well, I'm having a good time. Which makes me feel guilty too. How very English.

David Attenborough

AT the surface of the ocean live some microscopic animals
These are the zooplankton, phytoplankton are botanicals
Together these plankton form the marine food web base
Which makes them essential and hard to replace

Holoplankton spend lives within the zoo crowd
But meroplankton are larval stages of taxa more proud
Zooplankton have been collected for more than five years
Which makes IMOS, zooplankton pioneers

The collection occurred in Australia at nine sites
Which shows IMOS had tremendous insight
They collected primarily for their morphological analysis
But a proportion was saved for future analysts

While morphological study names species small and intact
Larval stages of most are less than exact
Fish and crustaceans are particularly challenging
Which means the composition of zooplankton is particularly baffling

THE samples used in this study are from Rottnest Isle
Which has sat on the southwest of WA for a while
The study set out with fifty-five samples
With molecular methods we strive to set the example

Our aim is to identify taxa and plot changes with time
To map seasonal and temperature variations would be sublime
To endeavour to do this we used eight primer sets
Some that exist and others original assets

The primers were designed mainly to target
Crustaceans and fish, and taxa not bought at a market
Illumina's MiSeq was the sequencer of choice
It's quick and precise, and gives sequences voice

THE eight primer sets named many phyla
A figure shows them all, sorry no monstrous hydra!
While we expected to see Arthropoda and Chordata
More interesting are Mollusca and Echinodermata
Over forty families were found; just by the Fish primer set
Included in a figure, they were all caught in the net
Over sixty taxa are represented just there
And thus they are added to the known Rottnest sphere

Another figure demonstrates OTU power
A seasonal analysis exhibits groupings and empowers
For an nMDS plot produced within R
Displays seasonal variation…Woo Hoo! Where's the bar!
The remaining primer sets yield a mass of results
Like crustaceans and molluscs not yet adults
Taxa that are morphologically invisible
Have been uncovered in an NGS miracle!

WE have s#*t loads of data yet to explore
But a lack of time and of space prevents us showing you more
Perhaps in a year you will come back to this spot
To see how we handled the data and produced more plots

This ongoing study will soon be expanded
To include all stations across Australia, curiosity demands it!
The aim is to provide a broad integral picture
Of these plankton communities and their internal mixture

CHAPTER – THREE

Marine environmental DNA biomonitoring reveals seasonal patterns in biodiversity and identifies ecosystem responses to anomalous climatic events

Science is about exploring, and the only way to uncover the secrets of the universe is to go and look.

Brian Cox

Science is the key to our future, and if you don't believe in science then you're holding everybody back.

Bill Nye

3.1 Prelude

CHAPTER Two focussed on an application of eDNA and metabarcoding; applying several assays to the scat of the endangered Australian sea lion (*Neophoca cinerea*). The work resulted in the methodologies being utilised in further collaborations. However the biodiversity revealed was limited to that sampled by the sea lion. To expand the range it was determined to source a more complex marine substrate.

Plankton communities are highly biodiverse and complex, which at the commencement of this research, had not been explored genetically to any great degree (Table 1.1). As a consequence, metabarcoding assays suitable for the task were mostly absent from the literature.

The Integrated Marine Observing System (IMOS; see Box 2.1) routinely collects plankton samples for morphological, biomass and genetic analysis. In 2014 access was granted to twenty plankton samples collected from the nine IMOS National Reference Stations (NRS) situated around Australia (Figure 4.1). Twelve samples were taken from the Rottnest Island site in Western Australia, across two summers (2011/12 and 2012/13), and the eight remaining samples originated from the other Australian NRS sites in January 2013. Morphological data, from plankton sampled contemporaneously, was also provided.

Supplied with samples and the appropriate preliminary morphological knowledge, the object was to design and implement a 'tool kit' and workflow to facilitate a better understanding of the intrinsic nature of a zooplankton community. This was initially achieved *in silico,* where sequences from related taxa, known to be within the samples, were downloaded in to Geneious (Kearse et al., 2012) and aligned to produce consensus sequences. These were then used to design the primers; the relatively conserved small DNA sequence pair that flanks the barcode of interest and form the basis of a sequencing assay. Over twenty novel assays were designed.

Box 3.1: Integrated Marine Observing System (IMOS)

IMOS is a consortium of institutions led by the University of Tasmania. It was established in 2007 for the purpose of observing long-term oceanic changes, boundary currents, inter-basin movements, continental shelf processes, climate patterns and extremes and the biological responses to these (Lynch et al., 2014). IMOS collects time-series and other data using an extensive range of observational equipment and from a variety of sites around Australia. This data is then made available to users such as Australian marine and climate scientists and their collaborators.

These new assays were tested—alongside several previously designed assays (Table S3.1)—using the provided samples. The biodiversity detected was vast. The assays were assessed for efficacy across several different zooplankton phyla to determine which combination would deliver a more comprehensive picture of zooplankton diversity.

Results from the pilot study were encouraging, so the project was expanded to examine the biodiversity of one site through time before addressing a wider temporal study. Accordingly, IMOS provided access to 55 mostly monthly samples from the Rottnest Island NRS, from circa 2010 to 2014 inclusive. This particular site was chosen for its well-documented heatwave period in 2011/2012 (Caputi, 2014, Pearce and Feng, 2013). The primary investigation was to determine whether, using the multi-gene metabarcoding method, temporal changes such as seasonal, annual and other potential climatic responses could be detected from this one site. Seven assays were selected from the pilot study 'tool kit' and a longer Universal 18S assay was also included. The eight assays produced over 400 individual datasets for analysis.

The results of this study are published in *PLOS Genetics* (Berry et al (2019) Vol. 15(2)); unique filtered sequence data from the study are uploaded to Data Dryad (https://doi:10.5061/dryad.sc673ds). The following chapter reproduces the paper as published

with the exception of formatting and referencing style. An as published version is available in the Appendix.

3.1.1 Acknowledgements

I thank the Integrated Marine Observing System (IMOS) for providing access to the samples and abiotic data used during this study. Supported by the Australian Government, IMOS is a national collaborative research infrastructure, which is operated by a consortium of institutions as an unincorporated joint venture, with the University of Tasmania as Lead Agent. An Australian Government Training Program Scholarship and the resources provided by the Pawsey Supercomputing Centre, which is funded by the Governments of Australia and Western Australia, also supported this work. M.B., E.S.H. and M.S. acknowledge the support of ARC Linkage projects (LP160100839 and LP160101508) to explore marine metabarcoding applications. I also thank Joey DiBattista for his editorial assistance.

3.1.2 Author Contributions

O.B., T.E.B., A.J.R., M.B. and M.S. conceived and designed the study. C.D. and A.J.R. facilitated access to the samples and abiotic data. T.E.B. and M.L.C. refined the approach and produced the data. B.S., T.E.B., E.S.H. and A.J.R designed and produced the statistical analysis. T.E.B., S.J., M.B., M.S. and B.S. discussed the results in preparation of the manuscript. All authors were involved in the final editing of the manuscript.

3.1.3 Author Declarations

The Authors declare they have no competing interests.

Marine environmental DNA biomonitoring reveals seasonal patterns in biodiversity and identifies ecosystem responses to anomalous climatic events

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3.2 Abstract

MARINE ecosystems are changing rapidly as the oceans warm and become more acidic. The physical factors and the changes to ocean chemistry that they drive can all be measured with great precision. Changes in the biological composition of communities in different ocean regions are far more challenging to measure because most biological monitoring methods focus on a limited taxonomic or size range. Environmental DNA (eDNA) analysis has the potential to solve this problem in biological oceanography, as it is capable of identifying a huge phylogenetic range of organisms to species level. Here we develop and apply a novel multi-gene molecular toolkit to eDNA isolated from bulk plankton samples collected over a five-year period from a single site. This temporal scale and level of detail is unprecedented in eDNA studies. We identified consistent seasonal assemblages of zooplankton species, which demonstrates the ability of our toolkit to audit community composition. We were also able to detect clear departures from the regular seasonal patterns that occurred during an extreme marine heatwave. The integration of eDNA analyses with existing biotic and abiotic surveys delivers a powerful new long-term approach to monitoring the health of our world's oceans in the context of a rapidly changing climate.

3.3 Author summary

ALL environments contain genetic remnants of the life they contain and support. For example, samples collected from the ocean contain biological material such as microscopic organisms, shed cells, excrement and saliva—the DNA from which reveals the surrounding marine biodiversity. Environmental DNA (eDNA) approaches have the ability to identify marine species that are notoriously difficult to identify using morphology alone. Here we develop, and apply, a DNA 'toolkit' to five years of samples collected from a single site in the Indian ocean. It is rare to find a temporal series of samples of this duration that are also suitable for DNA analysis. We show that eDNA techniques have the capacity to monitor ocean biology in fine detail. We demonstrate how the biological communities of plankton and fish respond to normal seasonal changes and, more importantly, to that of an uncharacteristic heatwave. The methods embodied in this paper are applicable to marine studies across the globe and, as such, pave the way for the design of long-term monitoring programs using eDNA.

3.4 Introduction

CHANGES in ocean temperatures, chemistry and currents are occurring faster now than at any time in human history (Beaugrand et al., 2002, Molinos et al., 2016). These changes will certainly impact the productivity in marine environments that is critical for social and economic wellbeing (Palmer, 2017). These impacts have driven the expansion of global efforts to monitor marine biota and track ecosystem health (Hays et al., 2005, Edwards et al., 2010, Beaugrand et al., 2002). Abiotic environmental data are already collected by various methods across all oceans (Lynch et al., 2014, Edwards et al., 2010), but thorough sampling of marine biota is far more restricted and challenging (Hays et al., 2005). Robust biomonitoring programs that link biological changes to the physio-chemical state of the oceans will help to identify ecological trends and predicting future trajectories.

Since 1931, the biomass and morphological species in zooplankton communities have been used extensively for oceanic biomonitoring (Warner and Hays, 1994). Zooplankton are the trophic link between phytoplankton and larger predators (Rice and Stewart, 2016). These highly diverse communities have been described as 'beacons of change' (Richardson, 2009), as their community composition is known to respond to fluctuations in both abiotic and biotic factors (Richardson, 2009, Hirai and Tsuda, 2015, Hays et al., 2005). Most zooplankton are ectothermic, so they are sensitive to temperature changes that affect their physical activity and physiology (Richardson, 2009). Many species are also fast growing and short-lived and so communities typically respond rapidly to environmental conditions (Richardson, 2009, Hirai and Tsuda, 2015, Hays et al., 2005, Kelly et al., 2016).

The importance of extended temporal sampling to describe changes within planktonic communities has long been recognised (Edwards et al., 2010, Mackas et al., 2012, Beaugrand et al., 2002, Chiba et al., 2006, Hays et al., 2005, Edwards and Richardson, 2004). A long-term analysis has the ability to define baselines and understand what is 'normal' for a community (Edwards et al., 2010) and provides a mechanism to gauge

ecosystem health (Kelly et al., 2016). There are several extended studies targeting zooplankton (Edwards et al., 2010, Hays et al., 2005, Edwards and Richardson, 2004, Chiba et al., 2006, Johnson et al., 2008, Molinero et al., 2005, Mackas et al., 2007, Beaugrand et al., 2002), yet these typically focus on a narrow range of taxa (Kelly et al., 2016, Mackas et al., 2012, Williams, 1984, Wiafe et al., 2016, Rakhesh et al., 2006, Beaugrand et al., 2002).

Morphological identification of zooplankton is time consuming and expensive (Edwards et al., 2010, Lindeque et al., 2013). Samples must be in good physical condition, particularly for taxonomic identifications reliant on the presence of fragile appendages. This problem is worst for easily damaged, soft-bodied phyla such as Cnidaria and Ctenophora (Purcell, 2012). Many marine animals, including fish and larger crustaceans, have a larval planktonic phase, and identification of larvae to species is difficult or impossible, even for skilled taxonomists (Bucklin et al., 2016, Markle and Frost, 1985, Lindeque et al., 2013). Morphological studies tend to overestimate the relative abundance of those taxa that are readily identified, but overlook a significant fraction of marine animal groups. Consequently there is growing recognition that morphology by itself will struggle to meet with the increasing need for holistic marine biomonitoring in conservation and management decisions (Harvey et al., 2017, Edwards et al., 2010).

Environmental DNA (eDNA) is transforming our ability to study marine biodiversity. Recent metabarcoding studies on eDNA extracted from water (Thomsen et al. 2012, Kelly et al., 2017, Stat et al., 2017), sediment (Haouchar et al., 2014), scat (Peters et al., 2014, Deagle et al., 2009, Boyer et al., 2015, Berry et al., 2017) and plankton (Lindeque et al., 2013, Deagle et al., 2017) demonstrate its capacity to profile a vast range of biota. While these studies focus strongly on spatial and community differences, the ability for eDNA to act as a long-term temporal biomonitoring tool is unexplored.

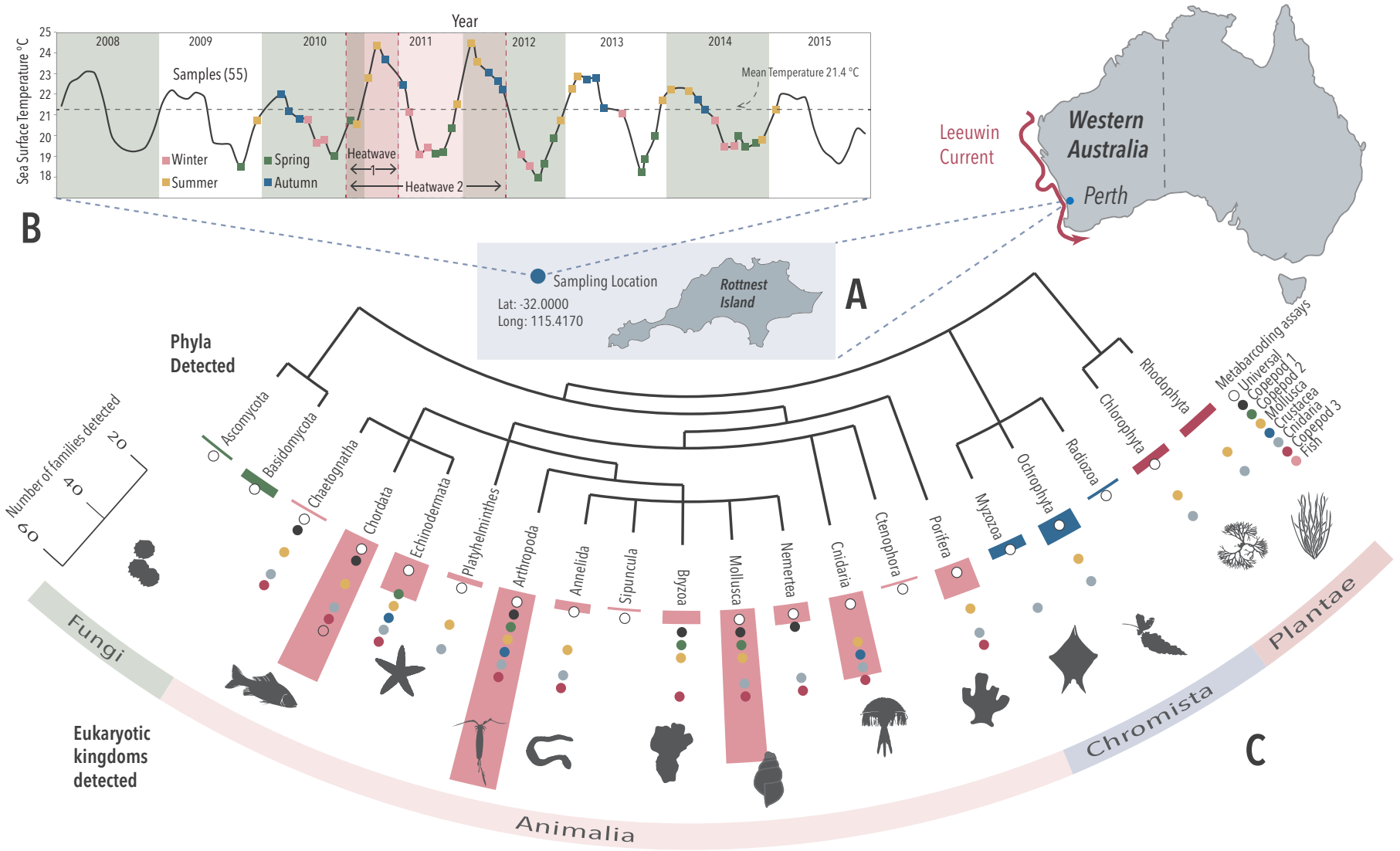
Environmental DNA is defined as a "*complex mixture of genomic DNA from many different organisms found in an environmental sample…* [including] *material resulting from filtering air or water, from sifting sediments, or from bulk samples*" (Taberlet et al., 2018). Here, due to historic sampling, we analyse eDNA purified from bulk zooplankton samples systematically collected monthly over five-years from a single ecologically significant site at Rottnest Island, Western Australia (Roberts et al., 2002; Figure 3.1A). This temporal window of sampling includes a "marine heatwave" anomaly that had significant impacts on the south Western Australian marine ecosystem (Pearce and Feng, 2013, Wernberg et al., 2016, Caputi et al., 2016). We test the capacity for eDNA metabarcoding to track potential biotic shifts, examine how eDNA signatures relate to abiotic variables, and lastly outline the value and practical implementation of multi-year eDNA programs

Shotgun DNA sequencing has been used for eDNA community analysis (Alberti et al., 2017) but it is cost prohibitive and dominated by prokaryote taxa (Stat et al., 2017). Single marker metabarcoding approaches have proven useful for biological monitoring, but their taxonomic focus has to be narrow because each assay is by definition limited in scope. Even supposedly "universal" DNA metabarcoding assays have proven inadequate to identify a comprehensive range of target taxa in our global oceans (Stat et al., 2017). To address the challenge of pinpointing a range of metazoan taxa, we developed a novel multi-gene (COI, 16S & 18S) metabarcoding 'toolkit' capable of working with both degraded and intact eDNA, and able to identify a wide variety of taxa found within zooplankton communities. We used three existing metabarcoding assays and designed five more (Table S3.1) to target a range of crustaceans, molluscs, fish and cnidarians known to be present at the reference site (Richardson et al., 2019)—a site that has been monitored using a variety of methods since 1951 (Lynch et al., 2014).

3.5 Results and discussion

OVERALL, while the majority of the eDNA extracted during this study originates from the plankton sampled (including larva and eggs), a small amount (impossible to quantify) would derive from sloughed cells or faecal material from larger organisms. From this total DNA more than four hundred distinct eukaryotic taxa were identified in this five-year study. These taxa were identified from more than nine million metabarcode sequences clustered into four thousand unique high abundance groups. Across all time points and assays, a total of 20 eukaryotic phyla were detected containing 245 families (Figure 3.1; Tables S3.2 – 3.7). Figure 3.1B also depicts the surface temperature and chronology of collection at the monitoring site. Most detections (70%) were within Arthropoda (including 62 families) and, of these, 87% were from Hexanauplia (including 24 families), the class that contains all copepods. The metabarcoding method employed here identified some of the gelatinous and larval zooplankton such as over 15 genera of hydrozoa and 50 genera of actinopterygii, many to species level. In practice, all assays, with the exception of the Fish assay, detected an extremely broad range of taxa. The Copepod 3 assay alone was responsible for over 1100 assignments across ten Animalia phyla; almost a quarter of all detections. It is, however, the integration of all assays that has revealed some of the breadth of biodiversity within this ecosystem over the five-year period. Had the study been limited to the 18S Universal assay, less than 70 assignments would have been made.

Figure 3.1: Extent of marine taxa revealed by eDNA from Rottnest Island. (A) 55 monthly plankton samples taken across five years (2009-2015) and an extreme heatwave event (B), which yielded 245 families of eukaryotic zooplankton across 20 phyla (C).


Year
Sea Surface Temperature °C
2008
2009
2010
2011
2012
2013
2014
2015
Samples (55)
Mean Temperature 21.4 °C
Winter
Spring
Summer
Autumn
Heatwave 1
Heatwave 2
B
Leeuwin Current
Western Australia
Perth
Sampling Location
Lat: -32.0000
Long: 115.4170
Rottnest Island
A
Phyla Detected
Number of families detected
Ascomycota
Basidomycota
Chaetognatha
Chordata
Echinodermata
Platyhelminthes
Arthropoda
Annelida
Sipuncula
Bryzoa
Mollusca
Nemertea
Cnidaria
Ctenophora
Porifera
Myzozoa
Ochrophyta
Radiozoa
Chlorophyta
Rhodophyta
Metabarcoding assays
Universal
Copepod 1
Copepod 2
Mollusca
Crustacea
Cnidaria
Copepod 3
Fish
Eukaryotic kingdoms detected
Fungi
Animalia
Chromista
Plantae
C

While Figure 3.1C showcases the taxa that our assays detected, more than 40% of the DNA sequences could not be reasonably assigned within a taxonomic framework. As a consequence of this problem, we applied a taxonomy-independent approach so that the analyses were not biased by the limitations of reference databases or the accuracy of the underpinning taxonomic framework. Operational Taxonomic Units (OTUs) enabled a more comprehensive exploration of the correlations between biotic and abiotic change over time.

3.5.1 Seasonal & Annual patterns

Biological monitoring at a single point in time is typically inadequate to describe total biodiversity or to explore changes in diversity over time. Collecting multiple time-stamped samples reveals greater total (gamma) biodiversity and allows measurement of beta diversity as a temporal change. For each assay, OTU biodiversity analysis involved both counting of the number of discrete OTUs—a measure hereafter referred to as "Richness"—and the presence/absence composition of the OTUs—referred to as "Assemblage". OTUs from each assay were examined independently so that comparisons were all made within the same experimental frameworks.

There are varying approaches for presenting eDNA metabarcoding data in terms of Assemblage and Richness. Some authors rarefy their data to normalise results for differing sequencing depth among libraries. We made the decision not to do this because sequence number and OTU accumulation curves had plateaued for each sample indicating that we had sampled the majority of the OTUs in each case (For example; Figure S3.1), Pearson's correlation tests showed there was no evidence to suggest a significant correlation between the number of sequences (i.e. sequencing depth) and the number of OTUs obtained for the 18S and 16S assays (Figure S3.2). However, sequencing depth and number of OTUs (Richness) were moderately correlated (R^2<0.522) for the COI assays (Figure S2). Nevertheless, as sequencing depth variation

is spread evenly across the samples (Figure S3.2), we consider it unlikely that Richness or Assemblage estimates are compromised by this data treatment.

Our initial analyses of eDNA (Table 3.1) demonstrated strong seasonality in the Assemblage from those assays that predominately detect meroplankton, including fish, molluscs and cnidarians. This seasonality was not reflected in Richness, with the exception of the Fish assay. A pairwise analysis between seasons (Table S3.8) indicated that the most consistent differences in Richness were detected between summer:winter, followed by spring:winter and spring:autumn. The least significant Assemblage changes were identified by the assays that predominantly detect holoplankton e.g. the Copepod assays. These detected no significant changes (after *post-hoc* correction) between winter:autumn, and summer:spring. These results provide a detailed example for multi-year marine biodiversity surveys based on eDNA.

Table 3.1: Significance of changes to the Operational Taxonomic Unit (OTU) Richness (a count of the number of OTUs in each sample) & Assemblage (the OTUs making up each sample) during different time periods within the five-year eDNA data including F statistics (F)— (PERMANOVA+ —Anderson et al., 2008).

Assay (Number of individual OTUs)	**OTU diversity test**	**Main tests 2010-2014**			**Main tests Before, During and After**	
		Month df (30,51)	**Season** df (15,51)	**Year** df (4,51)	**Heatwave 1** Nov 2010 – April 2011; df (2,54)	**Heatwave 2** Nov 2010 – May2012; df (2,54)
Cnidaria	Richness	- F=2.03	- F=0.93	- F=1.16	- F=1.03	- F=0.16
(246 OTUs)	Assemblage	- F=0.91	** F=1.38	** F=1.58	*** F=2.80	*** F=2.84
Copepod 1	Richness	- F=4.89	- F=1.21	** F=4.63	** F=6.60	* F=4.16
(171 OTUs)	Assemblage	* F=2.15	* F=1.41	*** F=1.99	*** F=3.28	*** F=3.64
Copepod 2	Richness	- F=2.76	- F=0.71	- F=1.50	- F=1.67	- F=0.34
(124 OTUs)	Assemblage	- F=0.10	- F=0.22	- F=1.48	- F=1.48	* F=2.01
Copepod 3	Richness	- F=2.76	- F=0.71	- F=1.50	- F=1.67	- F=0.34
(342 OTUs)	Assemblage	** F=2.31	*** F=1.48	- F=1.35	** F=1.94	** F=2.28
Crustacea	Richness	- F=0.55	- F=1.26	- F=1.96	- F=2.41	* F=3.59
(132 OTUs)	Assemblage	- F=0.86	* F=1.29	- F=1.15	- F=1.08	- F=1.32
Fish	Richness	- F=2.38	** F=3.49	- F=0.58	- F=0.09	- F=0.21
(87 OTUs)	Assemblage	* F=2.88	*** F=1.77	- F=0.79	- F=1.18	- F=0.85
Mollusca	Richness	- F=2.21	- F=1.27	- F=0.32	- F=0.57	- F=0.47
(345 OTUs)	Assemblage	- F=1.00	*** F=1.65	* F=1.39	*** F=2.41	*** F=2.29
Universal	Richness	- F=0.52	- F=0.96	- F=2.02	- F=0.40	- F=1.78
(97 OTUs)	Assemblage	- F=0.96	* F=1.34	- F=1.45	** F=2.08	*** F=2.45

Where *** is P ≤ 0.001, ** is P ≤ 0.01 & * is P≤ 0.05 & - is no significant changes

The Fish assay revealed strong seasonality in Richness and Assemblage (Figure 3.2). A pairwise analysis showed significant changes between all seasons for the Assemblage as well as Richness (Table S3.8), with exceptions being between adjacent seasons summer:spring and winter:autumn. Most fish are only present in the zooplankton community after broadcast spawning their eggs or during their pelagic larval phase, so these seasonal changes make biological sense (Markle and Frost, 1985). Seasonal fluctuations have been previously observed in fish using eDNA extracted from water (Stoeckle et. al., 2017, Sigsgaared et al., 2017). However, these studies were limited to durations of six and twelve months respectively. The current study provides additional and enduring evidence for the ability of eDNA to detect of seasonality over an extended period (5 years) and further incorporates a much broader range of biodiversity.

OTUs that characterise particular time periods were identified by *indval* analysis (Roberts, 2016). The strong seasonality in the Fish OTUs suggests that they might be driving significant differences identified in the seasonal *indval* analyses across all assays (Table S3.9), but this was not the case. Spring was characterised by a significant indicator matched to Labridae (a speciose fish family), but *Calcinus dapsiles* (a hermit crab) and *Evadne spinifera* (a water flea) were the summer's four top indicators. *Calcinus dapsiles* are only planktonic as larvae and only present seasonally, but *E. spinifera* is part of the plankton for its entire life.

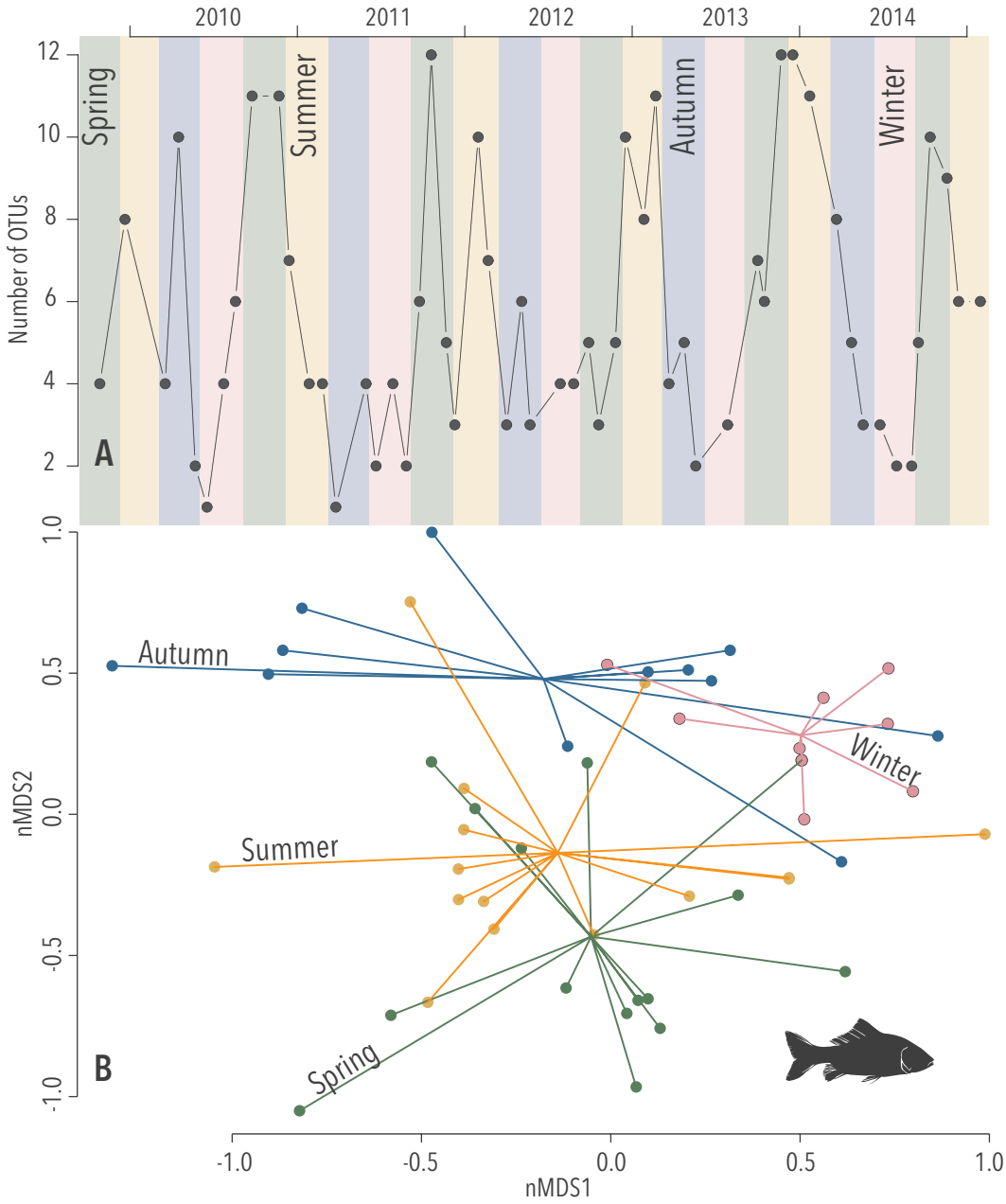


Figure 3.2: Seasonality in eDNA revealed by the Fish assay: (A) Number of Operational Taxonomic Units (OTUs) at each time point (Richness; $p < 0.001$) and (B) Diversity of OTUs as exhibited by a non-parametric multivariate analysis (Assemblage; $k = 3$, stress = 0.15, $p < 0.001$), the coloured lines extrude from the centroids of each season towards the variation of Assemblage in each sample.

Flaccisagitta enflata (a chaetognath or predatory arrow worm) and the copepods *Farranula gibbula* and *Centropages orsinii* were the most significant indicators for autumn. The copepods, *Canthocalanus pauper* and *Centropages furcatus* were found in winter. The genetic assignment of *C. orsinii* and *C. furcatus* are of interest as they are typically tropical species found in the Indian Ocean (ALA, 2016) indicating that they are likely to have been swept south by the warm water Leeuwin current (Figure 3.1A) in each year (Caputi et al., 1996). These indicator species analyses generate lists of target taxa that provide a more refined picture of seasonal changes in biodiversity—Table S3.9 lists all significant seasonally variable OTUs.

The years 2010 to 2014 showed changes in the Assemblage identified by several of the assays (Table 3.1); the pairwise analysis (Table S3.10) identified when these changes occurred. The OTUs that most strongly characterise each year are presented in Table S3.11. Six assays showed significant changes in Assemblage between 2010 and 2011 and each of the three subsequent years (Table S3.10). In particular, the Assemblage from Copepod 1, Mollusca and Cnidaria assays responded strongly. This pattern suggests a biotic regime shift in response to an environmental anomaly. Table S3.11 lists all significant yearly variable OTUs.

3.5.2 Biotic heatwave effects

The Rottnest Island area has global significance as it is situated within a site of high biodiversity that is largely endemic (Roberts et al., 2002). This sample set was particularly significant because it encompasses two uncharacteristic summer temperature extremes in 2011 and 2012. The WA heatwave was originally defined as occurring between November 2010 and April 2011 (Pearce and Feng, 2013). However, similarly high sea surface temperatures (SST) were recorded during the following year (Caputi etal., 2014, Pearce et al., 2016, Lenanton et al., 2017) (Figure 3.2B & Figure S3.3). In this study, periods for the heatwaves were: “Heatwave 1”, a five-month heatwave, as described in Pearce and Feng (2013); and “Heatwave 2”, which encompasses Heatwave

1 and extends across a 17-month period from November 2010 – May 2012 (Figure 3.1B). The Assemblage from most assays (except Crustacea, Fish) responded significantly to the designated heatwave periods (Table 3.1).

The most significant changes in the Assemblage were between the periods pre- and post-Heatwave 1 (Table S3.12). For Heatwave 2, significant differences were seen before, after, as well as during the thermal event (Table S3.12). Analyses of both heatwave periods suggest that there were significant, and potentially persistent, changes that occurred within the zooplankton communities as a result of these collective temperature anomalies. Only ongoing research will determine whether these changes are permanent, however, climate-mediated change has already been reported in the same study area. There Wernberg et al (2016) reported that a kelp dominated nearshore ecosystem shifted to a more tropicalised system containing seaweed turf.

The value of employing assays with different taxonomic specificities is shown by the lack of significant heatwave-induced Assemblage changes observed for some assays. No change was detected using the Crustacea and Fish assays, the taxa detected by these assays are generally long-lived with pelagic larval phases, so any significant change in these groups is likely to occur gradually and would only be detected with an even longer-term study. The Heatwaves had less significant effects on Richness, however the Copepod 1 and 3 assays demonstrated changes in Richness, particularly between before and after the thermal anomaly periods (Table S3.12).

The Copepod 1 assay illustrates the effects of Heatwaves 1 and 2 on the Assemblage and Richness (Figure 3.3). The Copepod 1 assay was designed *in silico* to focus on the genus *Triconia,* but, as is common in metabarcoding approaches, *in vitro,* the assay detects a much wider range of copepods as well as other arthropods.

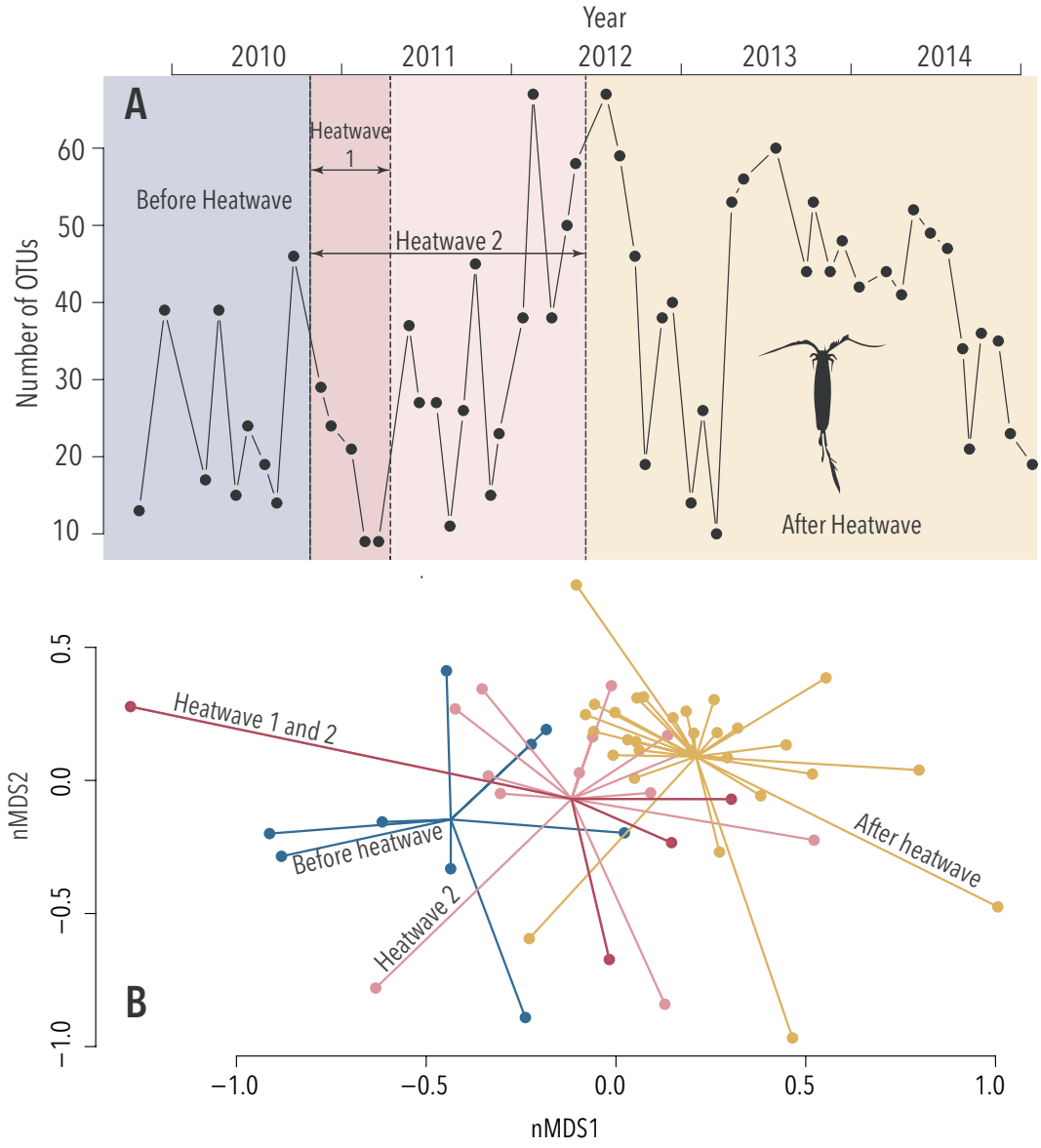


Figure 3.3: Heatwave effects revealed by Copepod 1 eDNA assay "Heatwave 1" and "Heatwave 2" are indicated. (A) Changes in the number of Operational Taxonomic Units (OTUs) over time (Richness: Heatwave 1; $p < 0.01$ & Heatwave 2; $p < 0.05$) and (B) changes in the diversity of the OTUs revealed by non-parametric multivariate analysis (Assemblage; $k = 3$, stress = 0.15, both $p < 0.001$), the coloured lines extrude from the centroids of each time period towards the variation in Assemblage from each sample.

OTUs characterising the periods defined by the heatwaves were identified by *indval* analysis. The OTUs corresponding to *Paracalanus indicus* (a copepod) and Pythiales (an order of water mould) are strong indicators for the 'before' periods (Tables S3.13 & S3.14). The Copepod 1 OTUs characterising the heatwave 'during' periods were significantly different; only ten OTUs (11%) overlap. The best indicator for Heatwave 1 was Hexanauplia (the class which contains all copepods); this OTU is also an indicator for Heatwave 2 (Tables S3.13 & S3.14). For the 'after' periods, nine OTUs are shared between them (15%). Nine anonymous copepod OTUs (15%) were strongly associated with the 'after' of both heatwave periods. This demonstrates the advantage of the OTU approach and provides an opportunity for taxonomists to link these sequences to the species that they provisionally represent.

These time-stamped metabarcoding data show, for the first time, that eDNA metabarcoding is able to track biotic shifts in response to seasonal and annual changes, as well as identify a known temperature anomaly that threatened global biodiversity hotspots on the west coast of Australia. This result has obvious implications for biomonitoring of oceans in the face of anthropogenic pressures including climate change, acidification, pollution, fishing and aquaculture impacts. The Assemblage and Richness data provided by eDNA metabarcoding can be integrated with other abiotic factors to develop a more holistic picture of how biomes respond to a variety of environmental factors.

3.5.3 Biological response to abiotic change

Biological samples analysed in this study were collected alongside complementary measurements of physical and chemical characteristics of the sampling site. Sea surface temperature (SST) and the concentrations of salinity and silicate (an important nutrient in oceans), were all important explanatory abiotic variables for both Richness and Assemblage across the majority of metabarcoding assays (Table 3.2). These variables

feature in either the 'best' or the most parsimonious alternative models for all of the assays used (Tables 3.2 and S3.15).

SST and salinity explained a large portion of the biological variation we observed. The assay most sensitive to the abiotic factors was Copepod 3; where SST, and concentrations of salinity, and silicate explained 22.7% of the variation in Assemblage, and SST and salinity concentration explained 39.2% of the variation in Richness (Table 3.2).

Richness increased significantly with warmer SST for most assays, with the exception of Copepod 1 and Copepod 2, which showed an insignificant negative relationship to SST (Table 3.2). Richness conversely decreased with increasing salinity for the Copepod, Crustacean, and Universal assays, but reacted positively in the Cnidaria, Fish, and Mollusca results. Silicate correlations had the opposite pattern, being positively correlated with Richness in the Copepod, Crustacean, and Universal results, but negatively correlated to Richness when measured against the Cnidaria, Fish, and Mollusca assays (Table 3.2). These results are likely due to an indirect link between the environmental variables to the zooplankton composition via direct links upon the phytoplankton (George et al., 2015). These results illustrate the different niches that zooplankton can exploit within an ecosystem. As one group of zooplankton find conditions uninhabitable and diminishes locally, another group will thrive within the niche.

Table 3.2: Relationship between sea surface temperature (SST) and abiotic factors, and OTU richness (the number of OTUs in each sample)—nbGLM (negative binominal Generalised Linear Model—Venables and Ripley, 2002)—and assemblage (what OTUs are in each sample)—DistLM (Distance based Linear Model —Anderson et al., 2008)—as indicated by each assay.

Assay used	OTU diversity test	Variable	SST	Salinity	Silicate	Nitrate	Phosphate	Ammonium	Best Model	
Cnidaria	Assemblage	P	**<0.001**	**<0.001**	**<0.001**	0.072	0.114	0.001	R^2	0.162
		R^2	**0.053**	**0.067**	**0.072**	0.028	0.026	0.044		
	Richness	P	**0.069**	0.061	**0.029**	0.842	0.809	0.700	R^2	0.112
		R^2	**0.056 (+)**	0.059 (+)	**0.078 (-)**	< 0.001 (-)	0.001 (+)	0.002 (-)		
Copepod 1	Assemblage	P	**0.030**	**0.002**	**< 0.001**	0.097	0.020	0.100	R^2	0.155
		R^2	**0.034**	**0.050**	**0.079**	0.028	0.036	0.028		
	Richness	P	0.953	**0.045**	0.308	0.478	0.376	0.245	R^2	0.067
		R^2	< 0.001 (-)	**0.067 (-)**	0.018 (+)	0.009 (+)	0.014 (+)	0.024 (+)		
Copepod 2	Assemblage	P	**0.021**	**< 0.001**	**< 0.001**	0.141	0.264	0.008	R^2	0.230
		R^2	**0.011**	**0.126**	**0.120**	0.027	0.022	0.049		
	Richness	P	0.428	**< 0.001**	**< 0.001**	0.146	0.172	0.011	R^2	0.309
		R^2	0.004 (-)	**0.255 (-)**	**0.204 (+)**	0.036 (+)	0.032 (+)	0.102 (+)		
Copepod 3	Assemblage	P	**0.011**	**<0.001**	**<0.001**	0.043	0.447	0.002	R^2	0.227
		R^2	**0.042**	**0.138**	**0.092**	0.034	0.018	0.053		
	Richness	P	**0.537**	**<0.0001**	0.007	0.252	0.561	**0.045**	R^2	0.392
		R^2	**0.007 (+)**	**0.305 (-)**	0.115 (+)	0.023 (+)	0.006 (+)	**0.067 (+)**		

Assay used	OTU diversity test	Variable	SST	Salinity	Silicate	Nitrate	Phosphate	Ammonium	Best Model	
Crustacea	Assemblage	P	**0.001**	**<0.001**	0.005	0.337	0.479	0.009	R^2	0.098
		R^2	**0.046**	**0.056**	0.038	0.021	0.019	0.037		
	Richness	P	**0.079**	0.246	0.799	0.104	0.629	**0.015**	R^2	0.183
		R^2	**0.052 (+)**	0.083 (-)	0.001 (+)	0.045 (+)	0 .004 (-)	**0.096 (+)**		
Fish	Assemblage	P	**0.001**	**<0.001**	0.007	0.006	**0.313**	0.002	R^2	0.147
		R^2	**0.056**	**0.064**	0.043	0.044	**0.022**	0.049		
	Richness	P	0.976	**0.007**	**0.005**	**0.016**	**0.679**	0.035	R^2	0.251
		R^2	<0.001 (+)	**0.121 (+)**	**0.127 (-)**	**0.098 (-)**	**0.003 (+)**	0.077 (-)		
Mollusca	Assemblage	P	**<0.001**	**<0.001**	**<0.001**	0.023	0.068	**<0.001**	R^2	0.197
		R^2	**0.057**	**0.101**	**0.081**	0.033	0.028	**0.051**		
	Richness	P	**0.058**	0.750	0.391	0.730	0.248	0.465	R^2	0.061
		R^2	**0.061 (+)**	0.013 (+)	0.019 (-)	0.002 (+)	0.024 (-)	0.010 (+)		
Universal	Assemblage	P	**0.045**	**0.001**	**0.001**	0.026	0.010	0.014	R^2	0.140
		R^2	**0.034**	**0.061**	**0.059**	0.038	0.043	0.043		
	Richness	P	**0.299**	**0.045**	0.165	0.286	**0.044**	0.578	R^2	0.212
		R^2	**0.019 (+)**	**0.067 (-)**	0.034 (+)	0.020 (+)	**0.068 (+)**	0.006 (+)		

Bolded type indicates abiotic variables that belong to the most parsimonious model as selected using the AIC
+ or – indicate the direction of the relationship

3.6 Conclusion

A recent editorial on marine monitoring (Borja et al., 2013) argued for a pressing need to make the shift from site-specific approaches to a functional, whole-sea system of monitoring. Here we show that eDNA metabarcoding is capable of responding to this challenge. Multi-year sample sets appropriate for eDNA analysis have not been previously available. Had this study been limited to a single point in time or even over the course of a year, the longer-term patterns of change would be missed. Our study included two 'marine heatwave' periods and these data demonstrated that, using an effective eDNA metabarcoding toolkit, ecologically significant trends can be identified in response to a known environmental perturbation.

The biodiversity detected by our multi-assay eDNA metabarcoding 'tool kit' was vast, and while many barcodes could be assigned within the existing taxonomic framework, almost as many could not. While it could be argued that indicator species/OTUs should perhaps be the primary focus for taxonomic scrutiny employing both morphology and genetics, it is clear that as databases and assays improve, so too will the power of eDNA to identify the taxa present in complex ecosystems like this one. The results highlighted both the importance of collecting time-stamped samples (i.e. environmental biobanks; Jarman et al., 2018) and the significance of multi-gene metabarcoding for the long-term monitoring of marine ecosystems. For example, had only the universal 18S marker been used, much of the genetic depth of information would have been lost. While the 18S markers are typically longer and produce results across a broad range of taxa, it is more conserved than other barcodes and often results must be confined to a family level of identification. The study illustrates the need to balance the cost of the multi-marker approach with the amount of data that can be generated. The future implications of this data are that eDNA will generate much-needed baseline biotic data, and identify disturbance gradients, recovery profiles and potential 'biotic tipping points'.

3.7 Materials and methods

3.7.1 Sampling

All sampling took place at the Rottnest Island National Reference Station (NRS), an Integrated Marine Observing System (IMOS; Lynch et al., 2014) site, Western Australia (Figure 3.1A). The site is situated at the midpoint of the sub-tropical zone of the Leeuwin current, approximately 20 km off the southwest coast of Western Australia. Abiotic sampling has occurred regularly at this site since 1951 and biological sampling by the IMOS program since 2008 (Lynch et al., 2014). The plankton sampling regime was instigated at this time and historically three separate monthly samples were taken; one for morphological analysis; one for biomass measurements and a third tow for later DNA analysis. We were provided access to these final samples.

Vertical plankton tows were taken on 55 occasions from October 2009 to January 2015, from the same site, in an almost regular monthly regime (Figure 3.1B). A 0.6 m wide, 3 m long drop net (Heron, 1982) with a100 μm mesh, which free falls at 1 ms-1, was dropped for 45 s. The seabed depth at the Rottnest Island sampling site is 50 m, so this sampling covered 90% of the water column. Plankton was collected on the downward fall; the motion of retrieval closes the net for the upward haul. The nets are washed, dried and stored between monthly sampling.

Samples were washed down and concentrated at the codend of the drop net and transferred into a sample jar using seawater. Samples were packed on ice until placed in long-term storage at -80°C immediately after return to the laboratory. Samples were later subsampled for this study and the sub-samples preserved at -20°C prior to DNA extraction.

3.7.2 DNA extraction

Each plankton sample was homogenised, using a hand-held blender (OMNI Tip Homogenizer) and a hard tissue probe. About 20 µL of the resulting slurry was digested and extracted using DNAeasy Blood and Tissue kit (Qiagen) following the tissue protocol and a 2 x 100 µL elution in AE buffer. An extraction control was created during this phase. Extracts were stored at -20 °C.

3.7.3 Metabarcoding assay design

Over 20 group-specific PCR amplicon metabarcode assays were tested for use in this study. Sequences used for *in silico* assay design were downloaded from the National Center for Biotechnology Information (NCBI) GenBank database (Benson et al., 2012). Database coverage was limited across all genes, so in most instances the cytochrome oxidase I (COI) gene provided the best option for metabarcoding.

Sequences were aligned in Geneious Version R8 and consensus sequences were derived from these alignments (Kearse et al., 2012). Sequences were examined for relatively conserved regions flanking 100-200bp hyper-variable targets (Figure S3.4). This examination resulted in the creation of several new metabarcoding assays. These assays, along with some that were previously described, were then tested against 20 plankton samples to determine which assays, when combined, produced the broadest coverage of taxa found within zooplankton (Table S3.16). From these, eight assays, including five targeting COI (predominately, three for different copepods and one each for molluscs and cnidarians), one targeting 18S rRNA ("universal") and two targeting16S rRNA (one each for actinopterygii and malacostraca), were selected for use (Table S3.1).

The 55 DNA extracts were assessed using qPCR for their response to each of the eight assays, which were applied to each sample's neat extract and two dilutions (1/10 and 1/100). Extraction, non-template and positive controls (where available) were included

for each assay. Each reaction comprised: 1 x Taq Gold buffer (Applied Biosystems [ABI], USA), 2 nM MgCl2 (ABI, USA), 0.4 mg/mL BSA (Fisher Biotec, Australia), 0.25 mM dNTPs (Astral Scientific, Australia), 0.4 µM each of forward and reverse primers (Integrated DNA Technologies, Australia), 0.6 µL of 1/10,000 SYBR Green dye (Life Technologies, USA), 1 U of *Taq* polymerase Gold (ABI, USA), 2 µL of DNA, and made up to 25 µL with PCR grade water. PCR conditions for all reactions included 95°C for 10 min followed by 50 cycles of 95°C for 30 sec, T_a (Table S1) for 30 sec and 72°C for 45 sec, with a final extension of 72°C for 10 min. All reactions were set up in an ultra-clean laboratory used for trace and environmental DNA.

3.7.4 Library builds & sequencing

Fusion tagged primers incorporating specific unique combinations of six to eight base pair MID (Multiplex IDentifier) tags, assay specific primers and Illumina adaptor sequences were assigned, in duplicate, to each DNA extract (and any negative control that produced a positive result during qPCR) in a single PCR step (giving a total of over 400 unique MID tagged combinations). Many samples are multiplexed within a single library and the MID tags allow for later separation and assignment of the individual sequences to their specific assays and samples. To prevent cross contamination within the NGS workflow, the MID tag primer combinations had not been used previously for marine samples and were not reused. Conditions for the fusion tagged PCR reactions were identical to the qPCR (above) and were carried out in duplicate, using the appropriate dilution determined by the qPCR. Reactions were monitored for efficient amplification by scrutinising qPCR dynamics. Tagged amplicons were combined in roughly equimolar concentrations to produce multiplexed sequencing libraries. On each library the fusion tags were not 'saturated', meaning that, while there are ten reverse tags to every forward tag, each run allowed for several unused forward and reverse combinations. If unused tag combinations are subsequently detected after sequencing, the tagging process is repeated to ensure there is no tag cross over. The libraries were then size-selected using a Pippin Prep (Sage Sciences, USA) instrument and quantified using a Lab Chip (PerkinElmer, USA). All sequencing was performed using Illumina's

MiSeq following the manufacturer's protocol with the exception of the use of custom sequencing primers and with 20 pM PhiX, on either a Standard or Nano flow cell and 300-500 cycle kits.

3.7.5 Taxonomic assignment

Sequences were assigned to the appropriate samples by their MID tags using Geneious R8 (Kearse et al., 2012). Initial filtering steps included ensuring the MID tags, gene specific primers and sequencing adapters, were all present in each sequence without error. Those sequences not matched were discarded from future analyses. The primers, adaptors and MID tags were removed from each of the sequences that passed these criteria, which were then filtered using a fastq filter (E_max > 0.5; USEARCH8; Edgar, 2010).

To increase the robustness of the data set, sequences were then separated into groups of unique sequences using USEARCH v8 (Edgar, 2010). Of these sequences, any group which contained < 1% of the total number of unique sequences was discarded—the filtered data are available for download on Data Dryad: doi:10.5061/dryad.sc673ds. This process, which may eliminate low abundance taxa, is conservative in that it ensures the removal of possible erroneous amplicons. Amplicons that passed the second filtering processes were queried against the National Center for Biotechnology Information (NCBI) GenBank nucleotide database (Benson et al., 2014) using BLASTn (Basic Local Alignment Search Tool; Altschul et al., 1990) with the default parameters and a reward of value of 1.

The search output files were imported into MEGAN v5 (METaGenome ANalyzer; Huson et al., 2011) and visualised using the LCA (lowest common ancestor) parameters: min bitscore 100.0, and reports restricted to the best 5% of matches. Taxonomic assignment was considered only when the entire length of the query sequence matched the reference database. Taxonomic hierarchy was determined using the World Register

of Marine Species (WoRMS, 2018). Negative controls were all found to be clear with the exception of the 18S Universal assay, which showed some fungal contamination.

3.7.6 Production of Operational Taxonomic Units (OTUs)

Clustering of similar sequences to produce OTUs was performed with USEARCH v8 (Edgar, 2010). The OTUs were formed using a 97% similarity threshold across all samples. The procedure also removed any potential chimeric sequences and any groups of unique sequences with an abundance of < 0.1% of the total number of unique sequences across all samples. Sequences discarded during this process were then mapped back on to existing OTUs to ensure the inclusion of all relevant data and those amplicons, which could not be mapped, were discarded. The OTUs were then assigned to the samples that they originated from and were converted to a presence/absence matrix. This approach also minimises any data misrepresentations as a result of potential unequal sequence amplification from marker choice or tag bias. The OTUs were statistically analysed in response to both temporal and abiotic factors.

3.7.7 Statistical analysis

Statistical analyses, were performed using PERMANOVA+ (Anderson et al., 2008) add on for Primer 7 (Clarke and Gorley, 2015) and R (R Core Team, 2015) with labdsv (Roberts, 2016), and vegan (Oksanen et al., 2016). The analyses were performed on the presence/absence OTU data matrix for the sequences obtained for each assay, thus allowing for all available genetic information to be taken into consideration. A total of 55 samples were used for analysis. The initial Pearson's correlation test of the number of sequences produced by each assay, at each time point, and the number of OTUs was performed in R (R Core Team, 2015).

To prevent the inclusion of 'outliers' that might skew the results, the sequences for each assay were filtered to remove any OTUs that occurred only once in the study and also any samples that contained only one OTU. The richness and assemblage (genetic diversity) data for each sample were then examined using multivariate methods (PERMANOVA; Anderson, 2001) to test time-based relationships such as heatwave, seasonality and inter-annual effects. Annual and seasonal effects were tested using a nested design with three factors: Year (fixed, 5 levels), Season (nested in Year, random), and Month (Nested in Season, random). Tests for heatwave effects were conducted using a single factor (fixed, either 5 month or 17 month heatwave window) with three levels (before, during, after). To illustrate these patterns, two-dimensional nonmetric multidimensional scaling (nMDS) plots were formed in R (package vegan).

The indicator species that were characteristic of years, seasons, and heatwave events were identified using indval analyses in R (package labdsv). The indval indicator value is calculated using a combination of the fidelity of an OTU to a time period and the frequency at which it occurs during that same time period. All pairwise comparisons were performed using PERMANOVA.

The role of abiotic variables in explaining variation in both the multivariate OTU assemblage, and the univariate OTU richness was analysed with linear models for each assay. Multivariate analysis was done using distance based linear models (DistLM) in PERMANOVA+. Bray-Curtis similarity matrices were constructed from the presence/absence OTU data. The abiotic variables sea surface temperature (SST) and concentrations of salinity, silicate, nitrate, phosphate, and ammonium were available for selection by the model. The 'best' selection procedure and the AIC selection criteria were used to select the model that best explained the variation in the OTU assemblage that was recorded for each assay. The best alternative models for each number of variables that were within 2 AIC of the selected model were also reported (Table S3.15).

Univariate OTU richness was analysed for each assay with generalised linear models (GLMs) fitted in R using the functions glm (R Core Team, 2015) and glm.nb (Venables and Ripley, 2002). The abiotic explanatory variables available were the same as those above. During analysis the distribution of the residuals of each model were plotted and examined to select the appropriate distribution. In all cases the negative binomial distribution with a log link was used (Zuur et al., 2009). The model with the lowest AIC was selected using the best of both forward and backward selection procedures. Models within 2AIC of the selected model were also reported. To aid in the interpretation of the relationship between each abiotic variable and the OTU assemblage composition and richness were also calculated and reported for each abiotic variable.

3.8 References

ALA. 2016. *Atlas of Living Australia website* [Online]. http://www.ala.org.au. [Accessed September 2016].

ALBERTI, A., POULAIN, J., ENGELEN, S., LABADIE, K., ROMAC, S., FERRERA, I., ALBINI, G., AURY, J. M., BELSER, C., BERTRAND, A., CRUAUD, C., DA SILVA, C., DOSSAT, C., GAVORY, F., GAS, S., GUY, J., HAQUELLE, M., JACOBY, E., JAILLON, O., LEMAINQUE, A., PELLETIER, E., SAMSON, G., WESSNER, M., GENOSCOPE TECHNICAL, T., ACINAS, S. G., ROYO-LLONCH, M., CORNEJO-CASTILLO, F. M., LOGARES, R., FERNANDEZ-GOMEZ, B., BOWLER, C., COCHRANE, G., AMID, C., HOOPEN, P. T., DE VARGAS, C., GRIMSLEY, N., DESGRANGES, E., KANDELS-LEWIS, S., OGATA, H., POULTON, N., SIERACKI, M. E., STEPANAUSKAS, R., SULLIVAN, M. B., BRUM, J. R., DUHAIME, M. B., POULOS, B. T., HURWITZ, B. L., TARA OCEANS CONSORTIUM, C., PESANT, S., KARSENTI, E. & WINCKER, P. 2017. Viral to metazoan marine plankton nucleotide sequences from the Tara Oceans expedition. *Sci Data,* 4**,** 170093.

ALTSCHUL, S. F., GISH, W., MILLER, W., MYERS, E. W. & LIPMAN, D. J. 1990. Basic local alignment search tool. *Journal of Molecular Biology,* 215(3)**,** 403-410.

ANDERSON, M., GORLEY, R. N. & CLARKE, R. K. 2008. *Permanova+ for Primer: Guide to Software and Statistical Methods*, Primer-E Limited.

ANDERSON, M. J. 2001. A new method for non-parametric multivariate analysis of variance. *Austral ecology,* 26(1)**,** 32-46.

BEAUGRAND, G., REID, P. C., IBANEZ, F., LINDLEY, J. A. & EDWARDS, M. 2002. Reorganization of North Atlantic marine copepod biodiversity and climate. *Science,* 296**,** 1692-1694.

BENSON, D. A., CLARK, K., KARSCH-MIZRACHI, I., LIPMAN, D. J., OSTELL, J. & SAYERS, E. W. 2014. GenBank. *Nucleic Acids Research,* 42**,** D32-D37.

BENSON, D. A., KARSCH-MIZRACHI, I., CLARK, K., LIPMAN, D. J., OSTELL, J. & SAYERS, E. W. 2012. GenBank. *Nucleic Acids Res,* 40**,** D48-53.

BERRY, T. E., OSTERRIEDER, S. K., MURRAY, D. C., COGHLAN, M. L., RICHARDSON, A. J., GREALY, A. K., STAT, M., BEJDER, L. & BUNCE, M. 2017. DNA metabarcoding for diet analysis and biodiversity: A case study using the endangered Australian sea lion (*Neophoca cinerea*). *Ecology & Evolution,* 7**,** 5435-5453.

BORJA, A., ELLIOTT, M., ANDERSEN, J. H., CARDOSO, A. C., CARSTENSEN, J., FERREIRA, J. G., HEISKANEN, A. S., MARQUES, J. C., NETO, J. M., TEIXEIRA, H., UUSITALO, L., UYARRA, M. C. & ZAMPOUKAS, N. 2013. Good Environmental Status of marine ecosystems: what is it and how do we know when we have attained it? *Marine Pollution Bulletin,* 76**,** 16-27.

BOYER, S., CRUICKSHANK, R. H. & WRATTEN, S. D. 2015. Faeces of generalist predators as 'biodiversity capsules': A new tool for biodiversity assessment in remote and inaccessible habitats. *Food Webs,* 3**,** 1-6.

BUCKLIN, A., LINDEQUE, P. K., RODRIGUEZ-EZPELETA, N., ALBAINA, A. & LEHTINIEMI, M. 2016. Metabarcoding of marine zooplankton: prospects, progress and pitfalls. *Journal of Plankton Research***,** fbw023.

CAPUTI, N., FLETCHER, W. J., PEARCE, A. & CHUBB, C. F. 1996. Effect of the Leeuwin Current on the Recruitment of Fish and Invertebrates along the Western Australian Coast. *Marine and Freshwater Research,* 47(2)**,** 147-155.

CAPUTI, N., JACKSON, G. AND PEARCE, A. 2014. The marine heat wave off Western Australia during the summer of 2010/11 - 2 years on. *Fisheries Research Report No 250.*

CAPUTI, N., KANGAS, M., DENHAM, A., FENG, M., PEARCE, A., HETZEL, Y. & CHANDRAPAVAN, A. 2016. Management adaptation of invertebrate fisheries to an extreme marine heat wave event at a global warming hot spot. *Ecology & Evolution,* 6(11)**,** 3583-3593.

CHIBA, S., TADOKORO, K., SUGISAKI, H. & SAINO, T. 2006. Effects of decadal climate change on zooplankton over the last 50 years in the western subarctic North Pacific. *Global Change Biology,* 12**,** 907-920.

CLARKE, K. & GORLEY, R. 2015. Getting started with PRIMER v7. *PRIMER-E: Plymouth, Plymouth Marine Laboratory*.

DEAGLE, B. E., CLARKE, L. J., KITCHENER, J. A., POLANOWSKI, A. M. & DAVIDSON, A. T. 2017. Genetic monitoring of open ocean biodiversity: An evaluation of DNA metabarcoding for processing continuous plankton recorder samples. *Molecular Ecology Resources*.

DEAGLE, B. E., KIRKWOOD, R. & JARMAN, S. N. 2009. Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces. *Molecular Ecology,* 18(9)**,** 2022-2038.

EDGAR, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics,* 26(19)**,** 2460-2461, 1367-4803.

EDWARDS, M., BEAUGRAND, G., HAYS, G. C., KOSLOW, J. A. & RICHARDSON, A. J. 2010. Multi-decadal oceanic ecological datasets and their application in marine policy and management. *Trends in Ecology & Evolution,* 25(10)**,** 602-610.

EDWARDS, M. & RICHARDSON, A. J. 2004. Impact of climate change on marine pelagic phenology and trophic mismatch. *Nature,* 430**,** 881-884.

GEORGE, J. A., LONSDALE, D. J., MERLO, L. R. & GOBLER, C. J. 2015. The interactive roles of temperature, nutrients, and zooplankton grazing in

controlling the winter–spring phytoplankton bloom in a temperate, coastal ecosystem, Long Island Sound. *Limnology and Oceanography,* 60**,** 110-126.

HAOUCHAR, D., HAILE, J., MCDOWELL, M. C., MURRAY, D. C., WHITE, N. E., ALLCOCK, R. J. N., PHILLIPS, M. J., PRIDEAUX, G. J. & BUNCE, M. 2014. Thorough assessment of DNA preservation from fossil bone and sediments excavated from a late Pleistocene–Holocene cave deposit on Kangaroo Island, South Australia. *Quaternary Science Reviews,* 84**,** 56-64.

HARVEY, J. B. J., JOHNSON, S. B., FISHER, J. L., PETERSON, W. T. & VRIJENHOEK, R. C. 2017. Comparison of morphological and next generation DNA sequencing methods for assessing zooplankton assemblages. *Journal of Experimental Marine Biology and Ecology,* 487**,** 113-126.

HAYS, G. C., RICHARDSON, A. J. & ROBINSON, C. 2005. Climate change and marine plankton. *Trends in Ecology & Evolution,* 20**,** 337-44.

HERON, A. C. 1982. A vertical free fall plankton net with no mouth obstructions. *Limnology and Oceanography,* 27(2)**,** 380-383.

HIRAI, J. & TSUDA, A. 2015. Metagenetic community analysis of epipelagic planktonic copepods in the tropical and subtropical Pacific. *Marine Ecology Progress Series,* 534**,** 65-78.

HUSON, D. H., MITRA, S., RUSCHEWEYH, H. J., WEBER, N. & SCHUSTER, S. C. 2011. Integrative analysis of environmental sequences using MEGAN 4, . *Genome Research.,* 21**,** 1552-1560.

JARMAN, S. N., BERRY, O. AND BUNCE, M. et al. 2018. The value of environmental DNA biobanking for long-term biomonitoring. *Nature Ecology & Evolution* 2(8): 1192-1193.

JOHNSON, C. L., LEISING, A. W., RUNGE, J. A., HEAD, E. J. H., PEPIN, P., PLOURDE, S. & DURBIN, E. G. 2008. Characteristics of *Calanus*

finmarchicus dormancy patterns in the Northwest Atlantic. *ICES Journal of Marine Science,* 65**,** 339-350.

KEARSE, M., MOIR, R., WILSON, A., STONES-HAVAS, S., CHEUNG, M., STURROCK, S., BUXTON, S., COOPER, A., MARKOWITZ, S., DURAN, C., THIERER, T., ASHTON, B., MEINTJES, P. & DRUMMOND, A. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics,* 28(12)**,** 1647-1649.

KELLY, P., CLEMENTSON, L., DAVIES, C., CORNEY, S. & SWADLING, K. 2016. Zooplankton responses to increasing sea surface temperatures in the southeastern Australia global marine hotspot. *Estuarine, Coastal and Shelf Science,* 180**,** 242-257.

KELLY, R. P., CLOSEK, C. J., O'DONNELL, J. L., KRALJ, J. E., SHELTON, A. O. & SAMHOURI, J. F. 2017 Genetic and manual survey methods yield different and complementary views of an ecosystem. *Frontiers in Marine Science,* 3:283

LENANTON, R. C. J., DOWLING, C. E., SMITH, K. A., FAIRCLOUGH, D. V. & JACKSON, G. 2017. Potential influence of a marine heatwave on range extensions of tropical fishes in the eastern Indian Ocean—Invaluable contributions from amateur observers. *Regional Studies in Marine Science,* 13**,** 19-31.

LINDEQUE, P.K., PARRY, H.E., HARMER, R.A., SOMERFIELD, P.J. & ATKINSON, A. 2013. Next generationn sequencing reveals the hidden diversity of zooplankton assemblages. *PLOS ONE,* 8, 11, e81327

LYNCH, T. P., MORELLO, E. B., EVANS, K., RICHARDSON, A. J., ROCHESTER, W., STEINBERG, C. R., ROUGHAN, M., THOMPSON, P., MIDDLETON, J. F., FENG, M., SHERRINGTON, R., BRANDO, V., TILBROOK, B., RIDGWAY, K., ALLEN, S., DOHERTY, P., HILL, K. & MOLTMANN, T. C. 2014. IMOS National Reference Stations: a continental-

wide physical, chemical and biological coastal observing system. *PLOS ONE,* 9**,** e113652.

MACKAS, D. L., BATTEN, S. & TRUDEL, M. 2007. Effects on zooplankton of a warmer ocean: Recent evidence from the Northeast Pacific. *Progress in Oceanography,* 75**,** 223-252.

MACKAS, D. L., GREVE, W., EDWARDS, M., CHIBA, S., TADOKORO, K., ELOIRE, D., MAZZOCCHI, M. G., BATTEN, S., RICHARDSON, A. J., JOHNSON, C., HEAD, E., CONVERSI, A. & PELUSO, T. 2012. Changing zooplankton seasonality in a changing ocean: Comparing time series of zooplankton phenology. *Progress in Oceanography,* 97-100**,** 31-62.

MARKLE, D. F. & FROST, L.-A. 1985. Comparative morphology, seasonality, and a key to planktonic fish eggs from the Nova Scotian shelf. *Canadian Journal of Zoology,* 63(2)**,** 246-257.

MOLINERO, J. C., IBANEZ, F., SOUISSI, S., CHIFFLET, M. & NIVAL, P. 2005. Phenological changes in the Northwestern Mediterranean copepods Centropages typicus and Temora stylifera linked to climate forcing. *Oecologia,* 145**,** 640-649.

MOLINOS, J. G., HALPERN, B. S., SCHOEMAN, D. S., BROWN, C. J., KIESSLING, W., MOORE, P. J., PANDOLFI, J. M., POLOCZANSKA, E. S., RICHARDSON, A. J. & BURROWS, M. T. 2016. Climate velocity and the future global redistribution of marine biodiversity. *Nature Climate Change,* 6(1)**,** 83-88.

OKSANEN, J., GUILLAUME BLANCHET, F., FRIENDLY, M., KINDT, R., LEGENDRE, P., MCGLINN, D., MINCHIN, P. R., O'HARA, R. B., SIMPSON, G. L., SOLYMOS, P., STEVENS, M. H. H., SZOECS, E. & WAGNER, H. 2016. vegan: Community Ecology Package. R package version 2.3-0. http://CRAN.R-project.org/package=vegan.

PALMER, C. P. 2017. Marine biodiversity and ecosystems underpin a healthy planet and social well-being. *UN Chronicle,* 54(2)**,** 59-61.

PEARCE, A., HUTCHINS, B., HOSCHKE, A. & FEARNS, P. 2016. Record high damselfish recruitment at Rottnest Island, Western Australia, and the potential for climate-induced range extension. *Regional Studies in Marine Science,* 8**,** 77-88.

PEARCE, A. F. & FENG, M. 2013. The rise and fall of the "marine heat wave" off Western Australia during the summer of 2010/2011. *Journal of Marine Systems,* 111-112**,** 139-156.

PETERS, K. J., OPHELKELLER, K., BOTT, N. J., DEAGLE, B.E., JARMAN, S.N. & GOLDSWORTHEY, S. D. 2014. Fine-scale diet of the Australian sea lion (*Neophoca cinerea*) using DNA-based analysis of faeces. *Marine Ecology,* 1-21

PURCELL, J. E. 2012. Jellyfish and ctenophore blooms coincide with human proliferations and environmental perturbations. *Annual Review of Marine Science,* 4**,** 209-235.

R CORE TEAM. 2015. *R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria* [Online]. Vienna, Austria. Available: http://www.R-project.org/. [Accessed 2016].

RAKHESH, M., RAMAN, A. V. & SUDARSAN, D. 2006. Discriminating zooplankton assemblages in neritic and oceanic waters: A case for the northeast coast of India, Bay of Bengal. *Marine Environmental Research,* 61(1)**,** 93-109.

RICE, E. & STEWART, G. 2016. Decadal changes in zooplankton abundance and phenology of Long Island Sound reflect interacting changes in temperature and community composition. *Marine Environmental Research,* 120**,** 154-165.

RICHARDSON, A. 2009. Plankton and climate. *Elements of Physical Oceanography: A derivative of the Encyclopedia of Ocean Sciences***,** 397.

RICHARDSON, A. J., URIBE-PALOMINO, J., SLOTWINSKI, A., COMAN, F., MISKIEWICZ, A. G., ROTHLISBERG, P. C., YOUNG, J. W. & SUTHERS, I. M. 2019. Coastal and marine zooplankton: identification, biology and

ecology. *Plankton: A guide to their ecology and monitoring for water quality.* Chapter 8, CSIRO publishing.

ROBERTS, C. M., MCCLEAN, C. J., VERON, J. E. N., HAWKINS, J. P., ALLEN, G. R., MCALLISTER, D. E., MITTERMEIER, C. G., SCHUELER, F. W., SPALDING, M., WELLS, F., VYNNE, C. & WERNER, T.B. 2002, Marine biodiversity hotspots and conservation priorities for tropical reefs. *Science,* 295 (5558) 1280-1284.

ROBERTS, D. W. 2016. labdsv: Ordination and Multivariate Analysis for Ecology. R package version 1.8-0.

SIGSGAARD, E. E., NIELSEN, I. B., CARL, H., KRAG, M. A., KNUDSEN, S. W., XING, Y., HOLM-HANSEN, T. H., MØLLER, P. R. & THOMSEN, P. F. 2017 Seawater environmental DNA reflects seasonality of a coastal fish community. *Marine Biology.* 164(6): 128

STAT, M., HUGGETT, M. J., BERNASCONI, R., DIBATTISTA, J. D., BERRY, T. E., NEWMAN, S. J., HARVEY, E. S. & BUNCE, M. 2017. Ecosystem biomonitoring with eDNA: metabarcoding across the tree of life in a tropical marine environment. *Sci Rep,* 7**,** 12240.

STOECKLE, M. Y., SOBOLEVA, L. & CHARLOP-POWERS, Z. 2017. Aquatic environmental DNA detects seasonal fish abundance and habitat preference in an urban estuary. *PLOS ONE,* 12**,** e0175186.

TABERLET, P., BONIN, A., ZINGER, L. & COISSAC, E. 2018. Environmental DNA for biodiversity research and monitoring. *Analysis of bulk samples.* Oxford University Press. Chapters 1 & 18

THOMSEN, P. F., KIELGAST, J., IVERSEN, L. L., MØLLER, P. R., RASMUSSEN, M., & WILLERSLEV, E. 2012. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. *PLOS ONE* 7(8):e41732

VENABLES, W. & RIPLEY, B. 2002. Random and mixed effects. *Modern applied statistics with S.* Springer. 271-300

WARNER, A. J. & HAYS, G. C. 1994. Sampling by the continuous plankton recorder survey. *Progress in Oceanography,* 34(2)**,** 237-256.

WERNBERG, T., BENNETT, S., BABCOCK, R. C., DE BETTIGNIES, T., CURE, K., DEPCZYNSKI, M., DUFOIS, F., FROMONT, J., FULTON, C. J., HOVEY, R. K., HARVEY, E. S., HOLMES, T. H., KENDRICK, G. A., RADFORD, B., SANTANA-GARCON, J., SAUNDERS, B. J., SMALE, D. A., THOMSEN, M. S., TUCKETT, C. A., TUYA, F., VANDERKLIFT, M. A. & WILSON, S. 2016. Climate-driven regime shift of a temperate marine ecosystem. *Science,* 353**,** 169.

WIAFE, G., DOVLO, E. & AGYEKUM, K. 2016. Comparative productivity and biomass yields of the Guinea Current LME. *Environmental Development,* 17, Supplement 1**,** 93-104.

WILLIAMS, R. 1984. Zooplankton of the Bristol Channel and Severn Estuary. *Marine Pollution Bulletin,* 15(2)**,** 66-70.

WoRMS Editorial Board: HORTON, T., KROH, A., AHYONG, S., BAILLY, N., BOURY-ESNAULT, N., BRANDÃO, S. N., COSTELLO, M. J., GOFAS, S., HERNANDEZ, F., MEES, J., PAULAY, G., POORE, G. C. B., ROSENBERG, G., DECOCK, W., DEKEYZER, S., LANSSENS, T., VANDEPITTE, L., VANHOORNE, B., VERFAILLE, K., ADLARD, R., ADRIAENS, P., AGATHA, S., AHN, K. J., AKKARI, N., ALVAREZ, B., ANDERSON, G., ANGEL, M., ARANGO, C., ARTOIS, T., ATKINSON, S., BANK, R., BARBER, A., BARBOSA, J. P., BARTSCH, I., BELLAN-SANTINI, D., BERNOT, J., BERTA, A., BIELER, R., BLANCO, S., BLASCO-COSTA, I., BLAZEWICZ, M., BOCK, P., BÖTTGER-SCHNACK, R., BOUCHET, P., BOXSHALL, G., BOYKO, C. B., BRAY, R., BREURE, B., BRUCE, N. L., CAIRNS, S., CAMPINAS BEZERRA, T. N., CÁRDENAS, P., CARSTENS, E., CHAN, B. K., CHAN, T. Y., CHENG, L.,

CHURCHILL, M., COLEMAN, C. O., COLLINS, A. G., CORBARI, L., CORDEIRO, R., CORNILS, A., COSTE, M., CRANDALL, K. A., CRIBB, T., CUTMORE, S., DAHDOUH-GUEBAS, F., DALY, M., DANELIYA, M., DAUVIN, J. C., DAVIE, P., DE BROYER, C., DE GRAVE, S., DE MAZANCOURT, V., DE VOOGD, N., DECKER, P., DECRAEMER, W., DEFAYE, D., D'HONDT, J. L., DIJKSTRA, H., DOHRMANN, M., DOLAN, J., DOMNING, D., DOWNEY, R., DRAPUN, I., ECTOR, L., EISENDLE-FLÖCKNER, U., EITEL, M., ENCARNAÇÃO, S. C. D., ENGHOFF, H., EPLER, J., EWERS-SAUCEDO, C., FABER, M., FEIST, S., FIGUEROA, D., FINN, J., FIŠER, C., FORDYCE, E., FOSTER, W., FRANK, J. H., et al. 2018. World Register of Marine Species (WoRMS). [cited 2018-04-11]. Available from: http://www.marinespecies.org

ZUUR, A., IENO, E., WALKER, N., SAVELIEV, A. & SMITH, G. 2009. Mixed effects models and extensions in ecology with R. New York: Springer. *574 p*.

3.9 Supplementary information

Table S3.1: The metabarcoding PCR assays used in this study

PCR assay	Primer set used	Primary target taxa	Gene	Primer sequence	Amplicon length (bp)	Reference	Assay Ta (°C)
Cnidaria	Cnidaria_F Cnidaria_R	Cnidarians	COI	5′ CATGATHTTYTTCWTDGTMATGCC 3′ 5′ GTYCAWCCWGTWCCWRCYCC 3′	~145	This study	52
Copepod 1	Triconia_F Triconia_R	Copepods	COI	5′ CAGGVTCTTTAYTWGGRGATG 3′ 5′ AAAATCTTATATTATTTARBCGRGG 3′	~131	This study	49
Copepod 2	Lucicutia_F Lucicutia_R	Copepods	COI	5′ CCHGAYATAGCTTTYCCHCG 3′ 5′ GAAAAAATTGCAAAATCTACDGATC 3′	~134	This study	52
Copepod 3	Acartia_F Acartia_R	Copepods	COI	5′ GGRGAYGATCARRTYTATAAYGT 3′ 5′ TTYATWCGWGGAAAHGCYATRTC 3′	~103	This study	50
Crustacea	Crust16S_ F (short) Crust16S_ R (short)	Crustaceans	16S rRNA	5′ GGGACGATAAGACCCTATA 3′ 5′ ATTACGCTGTTATCCCTAAAG 3′	~170	Berry et al. 2017	51
Mollusca	Limacina_F Limacina_R	Molluscs	COI	5′ TAATTGGNGGVTTTGGRAAYTG 3′ 5′ GTTCAHCCTRAYCCTRCNCC 3′	~118	This study	52
Fish	16s2R (degenerate) Fish16sF/D	Actinopterygii	16S rRNA	5′CGCTGTTATCCCTADRGTAACT 3′ 5′ GACCCTATGGAGCTTTAGAC 3′	~200	F- Deagle et al. 2007 R- Berry et al. 2017	54
Universal	18s_1F 18s_400R	Universal	18S rRNA	5′ GCCAGTAGTCATATGCTTGTCT 3′ 5′ GCCTGCTGCCTTCCTT 3′	~400	Pochon et al. 2013	52

Table 1: Primer sets used in this study: 'F' refers to the forward primer; 'R' refers to the reverse primer.

Table S3.2: Number of Arthropoda detections in Rottnest Island zooplankton samples by each assay.

Class	Order	Family	Genus	Species	Australia (ALA & Davies et al. 2014)	Rottnest (ALA, 2016)	Copepod 3	Cnidaria	Crust	Mollusca	Copepod 2	Copepod 1	18S
Branchiopoda - Diplostraca					Yes	Yes	1	0	0	0	0	0	0
Branchiopoda		Limnadiidae (ALA)			Yes	Yes	1	0	0	0	0	0	0
	Ctenopoda	Sididae	*Penilia*	*Penilia avirostris*	Yes	Yes	32	24	0	31	0	0	14
	Onychopoda	Podonidae	*Evadne*	*Evadne spinifera*	Yes	Yes	0	0	1	0	0	0	0
			Pseudevadne		Yes	Yes	0	0	0	4	0	0	0
				Pseudevadne tergestina	Yes	Yes	0	6	0	0	0	0	0
Hexanauplia	Calanoida				Yes	Yes	115	15	0	77	222	2	125
		Acartiidae			Yes	Yes	0	0	0	0	0	10	0
			Acartia		Yes	Yes	0	0	0	0	0	0	10
			Acartia (Acartia)	*Acartia (Acartia) danae*	Yes	Yes	20	0	0	1	0	0	1
				Acartia (Acartia) negligens	Yes	Yes	46	17	0	1	0	0	0
			Acartiella		No	No	0	0	0	0	32	0	0
		Aetideidae			Yes	Yes	0	0	0	0	6	0	25
			Aetideus	*Aetideus acutus*	Yes	No	0	0	0	0	4	0	0
			Gaetanus		Yes	No	0	0	0	0	0	0	17
		Augaptilidae	*Haloptilus*		Yes	Yes	0	0	0	0	0	0	4
				Haloptilus longicornis	Yes	Yes	0	0	0	0	2	0	0
		Calanidae			Yes	Yes	2	1	0	0	0	8	17
			Canthocalanus	*Canthocalanus pauper*	Yes	Yes	14	0	0	17	0	0	0
			Cosmocalanus		Yes	Yes	2	0	0	0	0	1	0
				Cosmocalanus darwinii	Yes	Yes	36	1	0	3	0	42	0
			Nannocalanus		Yes	Yes	1	0	0	5	0	3	0
			Neocalanus	*Neocalanus gracilis*	Yes	Yes	0	0	0	1	0	0	0
			Undinula	*Undinula vulgaris*	Yes	Yes	17	0	0	1	0	2	7
		Candaciidae			Yes	Yes	4	0	0	0	0	0	0
			Candacia		Yes	Yes	0	0	0	0	0	0	10

Class	Order	Family	Genus	Species	Australia (ALA & Davies et al. 2014)	Rottnest (ALA, 2016)	Copepod 3	Cnidaria	Crust	Mollusca	Copepod 2	Copepod 1	18S
				Candacia bipinnata	Yes	Yes	0	0	0	2	0	0	0
				Candacia bradyi	Yes	Yes	0	0	0	40	2	0	0
				Candacia catula	Yes	Yes	0	0	0	2	1	0	0
				Candacia discaudata	Yes	No	0	0	0	2	1	0	0
				Candacia pachydactyla	Yes	No	0	0	0	0	0	0	1
				Candacia simplex	Yes	No	1	0	0	0	0	0	0
				Candacia truncata	Yes	No	5	0	0	0	0	0	0
		Centropagidae			Yes	Yes	0	0	0	0	0	2	0
			Boeckella		Yes	Yes	0	0	0	2	0	0	0
			Centropages	*Centropages furcatus*	Yes	No	9	0	0	2	0	0	0
				Centropages orsinii	Yes	Yes	7	0	0	1	2	0	0
		Clausocalanidae	*Clausocalanus*		Yes	Yes	5	0	0	0	0	14	44
				Clausocalanus arcuicornis	Yes	Yes	6	0	0	0	0	0	0
				Clausocalanus farrani	Yes	Yes	0	0	0	1	0	34	0
				Clausocalanus furcatus	Yes	Yes	54	3	0	21	53	0	0
				Clausocalanus ingens	Yes	No	1	0	0	0	0	0	4
				Clausocalanus jobei	Yes	Yes	2	0	0	0	2	23	0
				Clausocalanus lividus	Yes	No	0	0	0	0	3	0	0
				Clausocalanus mastigophorus	Yes	Yes	0	0	0	0	1	0	0
				Clausocalanus minor	Yes	Yes	6	0	0	0	25	15	0
				Clausocalanus parapergens	Yes	No	1	0	0	0	23	4	0
				Clausocalanus pergens	Yes	No	1	0	0	0	0	0	0
			Ctenocalanus	*Ctenocalanus vanus*	Yes	Yes	16	0	0	0	7	32	0
		Eucalanidae			Yes	Yes	9	0	0	3	0	0	20
			Eucalanus		Yes	No	0	0	0	0	1	0	0
			Pareucalanus	*Pareucalanus attenuatus*	No	No	0	3	0	0	0	0	0

Class	Order	Family	Genus	Species	Australia (ALA & Davies et al. 2014)	Rottnest (ALA, 2016)	Copepod 3	Cnidaria	Crust	Mollusca	Copepod 2	Copepod 1	18S
				Pareucalanus sewelli	Yes	No	3	0	0	0	0	0	0
		Euchaetidae			Yes	Yes	0	0	0	0	0	1	0
			Euchaeta	*Euchaeta concinna*	Yes	No	4	0	0	0	0	2	0
				Euchaeta longicornis	No	No	0	0	0	1	0	0	0
				Euchaeta marina	Yes	No	0	0	0	9	11	0	0
		Lucicutiidae	*Lucicutia*	*Lucicutia flavicornis*	Yes	Yes	0	0	0	31	29	0	0
		Metridinidae	*Pleuromamma*	*Pleuromamma gracilis*	Yes	Yes	0	0	0	0	7	0	0
				Pleuromamma piseki	Yes	Yes	0	0	0	0	9	0	0
		Paracalanidae			Yes	Yes	0	0	0	0	16	8	1
			Acrocalanus		Yes	Yes	0	0	0	0	2	0	0
				Acrocalanus gracilis	Yes	Yes	11	0	0	0	2	1	0
				Acrocalanus monachus	Yes	No	0	0	0	0	0	0	10
			Bestiolina		Yes	Yes	38	0	0	29	0	0	0
			Calocalanus		Yes	Yes	25	0	0	1	48	1	0
				Calocalanus minutus	No	No	19	0	0	0	0	0	0
				Calocalanus pavo	Yes	Yes	11	0	0	14	3	8	0
				Calocalanus plumulosus	Yes	Yes	13	0	0	0	28	0	14
			Delibus		Yes	No	22	0	0	34	29	13	0
			Mecynocera	*Mecynocera clausi*	Yes	Yes	0	0	0	40	0	0	4
			Paracalanus		Yes	Yes	43	0	0	10	58	4	0
				Paracalanus aculeatus	Yes	Yes	45	0	0	0	0	0	0
				Paracalanus denudatus	Yes	No	0	0	0	0	21	29	0
				Paracalanus gracilis	No	No	0	0	0	0	17	0	0
				Paracalanus indicus	Yes	Yes	55	0	0	55	0	8	0
				Paracalanus nanus	No	No	41	0	0	4	30	5	0
				Paracalanus parvus	Yes	Yes	0	0	0	0	0	0	54
		Pontellidae	*Labidocera*	*Labidocera acuta*	Yes	No	0	2	0	1	0	0	0
				Labidocera minuta	Yes	Yes	33	0	0	2	0	0	0
			Pontellina	*Pontellina plumata*	Yes	No	1	11	0	1	0	0	0

Class	Order	Family	Genus	Species	Australia (ALA & Davies et al. 2014)	Rottnest (ALA, 2016)	Copepod 3	Cnidaria	Crust	Mollusca	Copepod 2	Copepod 1	18S
		Rhincalanidae	*Rhincalanus*		Yes	Yes	1	0	0	0	0	0	0
				Rhincalanus cornutus	Yes	No	0	0	0	1	0	0	0
		Scolecitrichidae	*Scolecithricella*		Yes	No	1	0	0	0	0	0	0
				Scolecithricella longispinosa	No	No	1	0	0	0	2	0	0
			Scolecithrix	*Scolecithrix danae*	Yes	Yes	17	0	0	0	0	0	0
		Subeucalanidae	*Subeucalanus*		Yes	Yes	5	0	0	10	0	0	4
				Subeucalanus mucronatus	Yes	No	2	1	0	3	1	0	0
				Subeucalanus pileatus	Yes	Yes	1	0	0	9	0	0	0
				Subeucalanus subtenuis	Yes	No	42	0	0	0	0	0	0
		Temoridae	*Temora*		Yes	Yes	0	0	0	0	0	0	5
				Temora discaudata	Yes	Yes	1	0	0	5	1	0	0
	Cyclopoida - Poecilostomatoida				Yes	Yes	0	0	0	3	2	2	0
	Cyclopoida	Corycaeidae	*Farranula*	*Farranula gibbula*	Yes	Yes	0	0	0	7	0	0	0
		Oncaeidae	*Oncaea*		Yes	Yes	0	0	0	2	0	8	0
				Oncaea mediterranea	Yes	Yes	0	0	0	6	0	10	0
				Oncaea scottodicarloi	Yes	No	0	0	0	3	0	12	0
				Oncaea waldemari	Yes	No	0	1	0	4	0	36	0
			Triconia		Yes	Yes	0	0	0	4	0	27	0
				Triconia minuta	No	No	0	0	0	0	0	9	0
				Triconia umerus	Yes	No	0	0	0	0	0	17	0
		Sapphirinidae			Yes	Yes	0	0	0	2	1	0	0
			Copila	*Copilia mirabilis*	Yes	Yes	0	0	0	12	0	0	0
			Sapphirina	*Sapphirina metallina*	Yes	No	0	0	0	1	1	0	0
	Harpacticoida	Euterpinidae	*Euterpina*	*Euterpina acutifrons*	Yes	Yes	0	0	0	0	0	0	1
	Lepadiformes	Lepadidae	*Conchoderma*	*Conchoderma virgatum*	Yes	No	2	2	0	1	0	0	0
Insecta	Coleoptera	Hydrophilidae			Yes	Yes	0	1	0	0	0	0	0

Class	Order	Family	Genus	Species	Australia (ALA & Davies et al. 2014)	Rottnest (ALA, 2016)	Copepod 3	Cnidaria	Crust	Mollusca	Copepod 2	Copepod 1	18S
Malacostraca	Amphipoda				Yes	Yes	0	0	0	1	0	0	0
		Ischyroceridae	*Jassa*	*Jassa slatteryi*	Yes	No	1	0	0	0	0	0	0
		Lestrigonidae	*Hyperietta*		Yes	Yes	2	0	0	0	0	0	0
				Hyperietta parviceps	No	No	0	0	0	2	0	0	0
			Hyperioides	*Hyperioides longipes*	Yes	No	1	0	0	0	0	0	0
				Hyperioides sibaginis	Yes	No	2	0	0	1	0	0	0
			Lestrigonus	*Lestrigonus bengalensis*	Yes	Yes	0	0	0	0	0	2	0
	Decapoda - Anomura				Yes	Yes	2	0	0	0	0	0	0
	Decapoda - Caridea				Yes	Yes	1	0	0	0	0	0	0
	Decapoda - Pilumnoidea				Yes	Yes	0	0	41	0	0	0	0
	Decapoda - Xanthoidea				Yes	Yes	1	0	0	0	0	0	0
	Decapoda				Yes	Yes	2	5	6	5	0	0	20
		Alpheidae			Yes	Yes	0	0	0	19	0	0	0
			Alpheus		Yes	Yes	0	0	0	0	0	0	2
		Axiidae	*Spongiaxius*	*Spongiaxius brucei*	Yes	No	0	0	0	0	0	0	1
		Callianassidae			Yes	Yes	0	0	0	0	0	0	1
			Trypaea	*Trypaea australiensis*	Yes	No	0	0	3	0	0	0	0
		Diogenidae	*Calcinus*	*Calcinus dapsiles*	Yes	Yes	10	0	9	0	0	0	0
		Epialtidae	*Menaethius*		Yes	No	0	0	4	0	0	0	0
		Hippolytidae			Yes	Yes	0	0	1	0	0	0	1
			Alope	*Alope orientalis*	Yes	Yes	0	0	5	0	0	0	0
			Hippolyte	*Hippolyte bifidirostris*	No	No	0	0	0	0	0	0	2
		Hymenosomatidae	*Halicarcinus*		Yes	Yes	0	0	1	1	0	0	1
		Luciferidae	*Belzebub*	*Belzebub intermedius*	No	No	5	0	0	0	0	0	0
			Lucifer		Yes	Yes	0	0	0	10	0	0	0
				Lucifer typus	Yes	No	3	0	0	0	0	0	0
		Lysmatidae	*Lysmata*	*Lysmata ternatensis*	No	No	0	0	1	0	0	0	0
		Palinuridae			Yes	Yes	1	0	0	0	0	0	0
			Panulirus	*Panulirus cygnus*	Yes	Yes	0	0	4	0	0	0	0

Class	Order	Family	Genus	Species	Australia (ALA & Davies et al. 2014)	Rottnest (ALA, 2016)	Copepod 3	Cnidaria	Crust	Mollusca	Copepod 2	Copepod 1	18S
		Pandalidae			Yes	Yes	0	0	0	0	0	0	1
		Panopeidae			Yes	No	1	0	0	0	0	0	0
			Eurytium		No	No	2	0	0	0	0	0	0
		Pasiphaeidae	*Leptochela*		Yes	Yes	0	0	0	0	0	0	3
		Penaeidae			Yes	Yes	0	0	2	0	0	0	0
		Polybiidae			Yes	Yes	0	0	3	0	0	0	0
			Liocarcinus	*Liocarcinus corrugatus*	Yes	Yes	2	0	0	0	0	0	0
		Portunidae	*Thalamita*		Yes	Yes	0	0	4	0	0	0	0
				Thalamita admete	Yes	No	0	0	3	0	0	0	0
		Processidae	*Nikoides*		Yes	No	0	0	2	0	0	0	0
				Nikoides danae	Yes	No	0	0	0	0	0	0	4
		Pseudoziidae			Yes	No	0	0	0	1	0	0	0
		Scyllaridae	*Crenarctus*	*Crenarctus bicuspidatus*	No	No	0	0	1	0	0	0	0
		Xanthidae			Yes	Yes	0	0	3	0	0	0	0
			Actaeodes		Yes	Yes	0	0	7	0	0	0	0
			Danielea	*Danielea noelensis*	No	No	0	0	1	0	0	0	0
			Nanocassiope		Yes	Yes	3	0	0	0	0	0	0
				Nanocassiope alcocki	Yes	No	1	0	11	0	0	0	0
			Paraxanthias	*Paraxanthias elegans*	Yes	No	0	0	2	0	0	0	0
	Euphausiacea				Yes	Yes	0	0	6	0	0	0	0
		Euphausiidae			Yes	Yes	0	0	0	0	0	0	2
			Euphausia		Yes	Yes	0	0	0	1	0	0	0
				Euphausia hemigibba	Yes	No	4	0	0	3	0	0	0
				Euphausia recurva	Yes	Yes	4	0	7	0	0	0	0
			Stylocheiron	*Stylocheiron carinatum*	Yes	No	0	1	0	0	0	0	0
	Sessilia				Yes	Yes	0	1	0	0	0	0	0
Ostracoda	Halocyprida	Halocyprididae			Yes	Yes	0	0	0	1	0	0	0
	Myodocopida	Cypridinidae			Yes	Yes	0	0	0	0	0	0	5

Table S3.3: Number of Chordata detections in Rottnest Island zooplankton samples by each assay.

Class	Order	Family	Genus	Species	In Australia (ALA, 2016)	Rottnest (ALA, 2016)	Copepod 3	Cnidaria	Fish	Mollusca	Copepod 1	18S
Actinopterygii	Anguilliformes	Congridae	*Gnathophis*		Yes	Yes	1	0	0	0	0	0
		Muraenidae			Yes	Yes	0	0	1	0	0	0
	Aulopiformes	Aulopidae			Yes	Yes	2	0	0	0	0	0
			Latropiscis	*Latropiscis purpurissatus*	Yes	Yes	0	0	1	0	0	0
		Notosudidae	*Scopelosaurus*		Yes	Yes	1	0	0	0	0	0
	Beryciformes	Berycidae	*Centroberyx*		Yes	Yes	0	0	1	0	0	0
				Centroberyx lineatus	Yes	Yes	1	0	0	1	0	0
	Clupiformes	Clupeidae	*Sardinops*	*Sardinops sagax*	Yes	Yes	31	30	37	31	0	0
			Spratelloides		Yes	Yes	0	0	1	0	0	0
		Dussumieriidae	*Etrumeus*		Yes	Yes	0	0	0	2	0	0
				Etrumeus teres	Yes	Yes	10	12	20	11	0	0
		Engraulidae	*Engraulis*		Yes	Yes	0	0	16	2	0	0
				Engraulis japonicus	No	No	8	0	0	0	0	0
	Gadiformes	Moridae			Yes	Yes	0	0	2	0	0	0
			Lotella	*Lotella rhacina*	Yes	Yes	2	0	1	0	0	0
	Myctophiformes				Yes	Yes	0	0	1	0	0	0
		Myctophidae			Yes	Yes	0	0	1	0	0	0
			Benthosema	*Benthosema fibulatum*	Yes	Yes	0	0	1	0	0	0
			Diaphus		Yes	Yes	1	0	6	0	0	0
			Lampanyctus	*Lampanyctus alatus*	Yes	Yes	1	0	0	0	0	0
			Notolychnus	*Notolychnus valdiviae*	Yes	Yes	0	0	1	0	0	0
			Scopelopsis	*Scopelopsis multipunctatus*	Yes	Yes	1	0	0	0	0	0
	Perciformes	Apogoninae			Yes	Yes	1	0	0	0	0	0
			Apogon	*Apogon doederleini*	Yes	Yes	1	1	0	1	0	0
		Arripidae	*Arripis*	*Arripis trutta*	Yes	Yes	0	0	1	0	0	0
		Blennidae	*Cirripectes*	*Cirripectes castaneus*	Yes	No	1	0	0	0	0	0
		Carangidae	*Pseudocaranx*	*Pseudocaranx wrighti*	Yes	Yes	1	0	2	0	0	0
			Trachurus		Yes	Yes	0	0	8	1	0	0
		Cheilodactylidae	*Nemadactylus*		Yes	Yes	0	0	2	0	0	0

Class	Order	Family	Genus	Species	In Australia (ALA, 2016)	Rottnest (ALA, 2016)	Copepod 3	Cnidaria	Fish	Mollusca	Copepod 1	18S
		Emmelichthyidae	*Plagiogeneion*		Yes	Yes	1	0	0	0	0	0
				Plagiogeneion rubiginosum	Yes	Yes	0	0	1	0	0	0
		Gerreidae	*Parequula*		Yes	Yes	0	0	1	0	0	0
				Parequula melbournensis	Yes	Yes	0	0	3	0	0	0
		Glaucosomatidae	*Glaucosoma*	*Glaucosoma hebraicum*	Yes	Yes	0	0	1	0	0	0
		Gobiidae			Yes	Yes	0	0	1	0	0	0
		Haemulidae			Yes	Yes	0	0	1	0	0	0
		Kyphosidae	*Kyphosus*		Yes	Yes	0	0	1	0	0	0
		Labridae			Yes	Yes	5	0	4	0	0	0
			Austrolabrus	*Austrolabrus maculatus*	Yes	Yes	0	0	16	0	0	0
			Choerodon		Yes	Yes	0	0	1	0	0	0
			Coris		Yes	Yes	0	0	2	0	0	0
				Coris auricularis	Yes	Yes	1	0	0	0	0	0
			Halichoeres	*Halichoeres brownfieldi*	Yes	Yes	0	0	4	0	0	0
			Labroides	*Labroides dimidiatus*	Yes	Yes	0	0	1	0	0	0
			Notolabrus		Yes	Yes	1	0	0	0	0	0
			Ophthalmolepis	*Ophthalmolepis lineolata*	Yes	Yes	0	0	1	0	0	0
			Pictilabrus		Yes	Yes	0	0	15	0	0	0
			Pseudolabrus	*Pseudolabrus biserialis*	Yes	Yes	0	0	1	0	0	0
			Thalassoma		Yes	Yes	0	0	1	0	0	0
		Mullidae			Yes	Yes	0	0	0	11	0	0
			Upeneichthys		Yes	Yes	0	0	2	0	0	0
				Upeneichthys stotti	Yes	Yes	0	0	10	0	0	0
				Upeneichthys vlamingii	Yes	Yes	0	0	0	1	0	0
		Odacidae			Yes	Yes	0	0	3	0	0	0
			Heteroscarus	*Heteroscarus acroptilus*	Yes	Yes	3	0	0	0	0	0
			Odax	*Odax acroptilus*	Yes	Yes	0	0	3	0	0	0
		Opistognathidae			Yes	Yes	0	0	1	0	0	0
		Pempheridae	*Parapriacanthus*		Yes	Yes	0	0	3	0	0	0
				Parapriacanthus elongatus	Yes	Yes	0	0	11	0	0	0

Class	Order	Family	Genus	Species	In Australia (ALA, 2016)	Rottnest (ALA, 2016)	Copepod 3	Cnidaria	Fish	Mollusca	Copepod 1	18S
		Pinguipedidae	*Parapercis*	*Parapercis ramsayi*	Yes	Yes	0	0	1	0	0	0
		Pomacentridae	*Chromis*		Yes	Yes	4	0	5	0	0	0
				Chromis notata	No	No	4	0	4	0	0	0
		Scaridae	*Scarus*	*Scarus chameleon*	Yes	Yes	1	0	0	0	0	0
		Scombridae	*Scomber*		Yes	Yes	1	0	1	0	0	0
		Sillaginidae	*Sillaginodes*	*Sillaginodes punctatus*	Yes	Yes	0	0	2	0	0	0
			Sillago		Yes	Yes	1	0	1	0	0	0
				Sillago bassensis	Yes	Yes	1	0	1	0	0	0
		Sparidae			Yes	Yes	0	0	1	0	0	0
	Scorpaeniformes				Yes	Yes	0	0	1	0	0	0
		Neosebastidae	*Neosebastes*		Yes	Yes	0	0	2	0	0	0
		Platycephalidae	*Leviprora*	*Leviprora inops*	Yes	Yes	0	0	0	1	0	0
			Platycephalus		Yes	Yes	0	0	5	0	0	0
			Thysanophrys	*Thysanophrys cirronasa*	Yes	Yes	0	0	0	1	0	0
		Triglidae	*Lepidotrigla*		Yes	Yes	0	0	2	0	0	0
	Stomiiformes	Gonostomatidae	*Cyclothone*		Yes	Yes	0	0	1	0	0	0
		Phosichthyidae	*Vinciguerria*		Yes	Yes	3	0	0	1	0	0
	Tetraodontiformes	Monacanthidae			Yes	Yes	3	0	3	0	0	0
			Acanthaluteres		Yes	Yes	0	1	0	0	0	0
				Acanthaluteres vittiger	Yes	Yes	0	0	4	0	0	0
			Eubalichthys	*Eubalichthys mosaicus*	Yes	Yes	2	1	1	0	1	0
			Nelusetta	*Nelusetta ayraudi*	Yes	Yes	1	0	1	0	0	0
			Scobinichthys	*Scobinichthys granulatus*	Yes	Yes	0	0	1	0	0	0
Amphibia	Anura	Ranidae			Yes	No	1	0	0	0	0	0
Appendicularia	Copelata	Oikopleuridae	*Oikopleura*		Yes	No	0	0	0	0	0	1
				Oikopleura dioica	Yes(Thompson, 1948)	No	0	0	0	0	0	10
Ascidiacea	Phlebobrancia	Perophoridae			Yes	Yes	0	0	0	0	0	1
	Stolidobranchia	Pyuridae	*Herdmania*	*Herdmania momus*	Yes	Yes	0	0	0	0	0	1
Thaliacea	Salpida	Salpidae			Yes	Yes	0	0	0	0	0	1
			Brooksia	*Brooksia rostrata*	Yes	No	0	0	0	0	0	1

Class	Order	Family	Genus	Species	In Australia (ALA, 2016)	Rottnest (ALA, 2016)	Copepod 3	Cnidaria	Fish	Mollusca	Copepod 1	18S
			Cyclosalpa		Yes	No	0	0	0	0	0	1
			Thalia		Yes	No	0	0	0	0	0	8
	Doliolida	Doliolidae			Yes	Yes	0	0	0	0	0	1
			Doliolum	*Doliolum nationalis*	Yes(Thompson, 1948)	No	1	0	0	0	0	0

Table S3.4: Number of Mollusca detections in Rottnest Island zooplankton samples by each assay.

Class	Order	Family	Genus	Species	In Australia (ALA, 2016)	Rottnest (ALA, 2016)	Copepod 3	Cnidaria	Mollusca	Copepod 2	Copepod 1	18S
Bivalvia	Ostreida	Ostreidae			Yes	Yes	0	1	0	0	0	0
		Pinnidae			Yes	Yes	0	0	1	0	0	0
	Pterioida (ALA)				Yes	No	0	0	2	0	0	0
Cephalopoda	Decapodiformes	Idiosepiidae			Yes	Yes	0	0	1	0	0	0
Gastropoda - Hypsogastropoda					Yes	Yes	0	0	0	0	14	0
Gastropoda					Yes	Yes	0	0	0	0	6	4
		Acteonidae	*Pupa*		Yes	Yes	0	0	1	0	0	0
		Aplustridae	*Micromelo*	*Micromelo undatus*	Yes	No	0	1	0	0	1	0
		Lepetidae			Yes	No	1	0	0	0	0	0
		Lottiidae			Yes	Yes	1	0	0	0	0	0
		Pyramidellidae	*Cingulina*		Yes	Yes	0	0	1	0	0	0
	Anaspidea	Aplysiidae			Yes	Yes	0	0	3	0	0	0
			Aplysia	*Aplysia gigantea*	Yes	Yes	0	0	2	0	0	0
			Dolabella	*Dolabella auricularia*	Yes	Yes	0	1	0	0	0	0
	Cephalaspidea	Bullidae	*Bulla*	*Bulla quoyii*	Yes	Yes	0	0	4	0	0	0
	Caenogastropoda				Yes	Yes	0	0	0	0	5	2
		Cerithiidae	*Cacozeliana*	*Cacozeliana granarium*	Yes	Yes	0	0	24	0	1	0
		Newtoniellidae	*Ataxocerithium*		Yes	Yes	10	0	0	5	14	0
		Potamididae			Yes	Yes	1	0	0	0	0	0
	Cephalaspidea	Aglajidae			Yes	Yes	0	0	1	0	0	2
	Cycloneritimorpha				Yes	Yes	0	0	0	2	0	0
		Neritidae			Yes	Yes	3	0	0	0	0	0
	Littorinimorpha				Yes	Yes	0	0	0	9	3	0
		Bursidae			Yes	Yes	1	0	0	0	0	0
			Bursina		Yes	No	1	0	0	0	0	0
		Calyptraeidae			Yes	Yes	1	0	0	0	0	0

Class	Order	Family	Genus	Species	In Australia (ALA, 2016)	Rottnest (ALA, 2016)	Copepod 3	Cnidaria	Mollusca	Copepod 2	Copepod 1	18S
		Cassidae			Yes	Yes	0	0	1	0	0	0
			Semicassis		Yes	Yes	1	0	0	0	0	0
		Cypraeidae	*Erosaria*	*Erosaria cernica*	Yes	Yes	0	2	0	0	0	0
			Staphylaea	*Staphylaea limacina*	Yes	No	0	0	0	0	1	0
		Hipponicidae	*Antisabia*	*Antisabia foliacea*	Yes	Yes	1	0	4	0	0	0
		Naticidae	*Conuber*		Yes	Yes	0	0	0	0	1	0
		Rissoinidae	*Phosinella*	*Phosinella clathrata*	Yes	No	0	0	0	0	0	1
		Strombidae			Yes	Yes	0	0	0	1	0	0
			Canarium	*Canarium mutabile*	Yes	Yes	0	0	0	0	1	0
		Triviidae	*Ellatrivia*	*Ellatrivia merces*	Yes	Yes	0	0	0	0	1	0
	Neogastropoda				Yes	Yes	0	0	1	2	6	1
		Buccinidae			Yes	Yes	2	0	2	0	0	0
			Lirabuccinum		No	No	8	0	0	0	0	0
				Lirabuccinum dirum	No	No	1	0	0	0	0	0
		Columbellidae			Yes	Yes	1	0	2	0	0	0
		Conidae			Yes	Yes	1	0	0	0	0	0
			Conus	*Conus*	Yes	Yes	0	0	0	0	2	0
				Conus klemae	Yes	Yes	1	0	1	0	2	0
		Mitridae	*Mitra*	*Mitra cucumerina*	Yes	No	0	0	0	0	1	0
		Muricidae			Yes	Yes	23	0	1	1	1	0
			Cronia		Yes	Yes	0	0	1	2	0	0
			Dicathais	*Dicathais orbita*	Yes	Yes	0	0	2	0	3	0
	Nudibranchia				Yes	Yes	0	10	2	1	2	0
		Chromodorididae			Yes	Yes	0	1	0	0	0	0
			Ceratosoma	*Ceratosoma amoenum*	Yes	Yes	0	2	1	0	0	0

Class	Order	Family	Genus	Species	In Australia (ALA, 2016)	Rottnest (ALA, 2016)	Copepod 3	Cnidaria	Mollusca	Copepod 2	Copepod 1	18S
			Chromodoris	*Chromodoris striatella*	Yes	No	0	6	1	0	0	0
		Dotidae	*Doto*		Yes	Yes	0	0	1	0	0	0
		Eubranchidae			Yes	No	0	0	0	0	1	0
		Tergipedidae			Yes	Yes	0	0	0	0	2	0
	Pleurobranchomorpha	Pleurobranchidae			Yes	Yes	0	0	1	0	0	0
			Pleurobranchus	*Pleurobranchus hilli*	Yes	Yes	0	3	0	0	0	0
	Sacoglossa				Yes	Yes	0	1	0	0	0	0
		Limapontiidae			Yes	Yes	0	0	1	0	0	0
	Thecosomata				Yes	Yes	0	1	1	0	0	0
		Creseidae			Yes	No	0	0	11	0	0	0
			Creseis		Yes	No	0	1	18	0	0	4
				Creseis clava	No	No	0	0	1	0	0	0
		Cuvierinidae	*Cuvierina*		Yes	Yes	0	1	0	0	0	0
Polyplacophora	Chitonida	Mopaliidae			Yes	Yes	2	0	0	0	0	0

Table S3.5: Number of Cnidaria detections in Rottnest Island zooplankton samples by each assay.

Class	Order	Family	Genus	Species	In Australia (ALA, 2016)	Rottnest (ALA, 2016)	Copepod 3	Cnidaria	Crust	Mollusca	18S
Anthozoa	Actiniaria				Yes	Yes	3	0	0	2	1
	Pennatulacea	Virgulariidae	*Stylatula*	*Stylatula elongata*	No	No	0	1	0	1	0
	Scleractinia	Caryophylliidae			Yes	Yes	1	0	0	0	0
	Zoantharia				Yes	Yes	1	1	0	3	0
		Zoanthidae			Yes	Yes	0	1	0	1	0
		Microzoanthidae	*Microzoanthus*		Yes	No	0	2	0	0	0
Hydrozoa					Yes	Yes	0	1	0	0	0
	Anthoathecata				Yes	Yes	0	9	0	0	7
		Oceaniidae	*Turritopsis*		Yes	No	0	0	0	11	0
		Pennariidae	*Pennaria*		Yes	Yes	0	1	0	0	0
	Leptothecata				Yes	Yes	0	7	0	0	0
		Campanulariidae			Yes	Yes	0	9	0	1	0
			Clytia		Yes	Yes	0	1	0	4	0
			Obelia		Yes	Yes	0	1	0	0	0
		Eirenidae	*Eirene*		Yes	No	0	0	0	1	0
		Plumulariidae			Yes	Yes	0	0	0	1	0
		Thyroscyphidae	*Thyroscyphus*	*Thyroscyphus ramosus*	No	No	0	0	0	0	1
	Narcomedusae	Cuninidae	*Cunina*		Yes	No	0	0	0	1	0
	Siphonophorae				Yes	Yes	0	3	0	0	2
		Abylidae	*Abylopsis*	*Abylopsis eschscholtzii*	No	No	0	1	0	0	0
			Bassia	*Bassia bassensis*	No	No	0	17	0	0	0
		Agalmatidae			Yes	No	0	2	0	2	0
			Athorybia	*Athorybia rosacea*	No	No	0	1	0	0	0
			Nanomia		Yes	No	0	3	0	0	0
				Nanomia bijuga	No	No	2	0	0	0	1
		Cordagalmatidae	*Cordagalma*	*Cordagalma ordinatum*	No	No	0	0	0	0	1
		Diphyidae			Yes	Yes	0	2	0	0	0
			Lensia		No	No	0	2	0	0	0
				Lensia campanella	No	No	1	0	0	0	0

Class	Order	Family	Genus	Species	In Australia (ALA, 2016)	Rottnest (ALA, 2016)	Copepod 3	Cnidaria	Crust	Mollusca	18S
				Lensia subtiloides	No	No	0	4	0	21	0
		Sphaeronectidae	*Sphaeronectes*	*Sphaeronectes koellikeri*	No	No	0	33	0	4	0
	Trachymedusae	Geryoniidae			Yes	Yes	0	0	1	0	2
			Geryonia	*Geryonia proboscidalis*	Yes	No	1	0	0	0	0
			Liriope		Yes	No	0	2	0	0	0
				Liriope tetraphylla	Yes	No	27	0	0	0	0
		Rhopalonematidae	*Aglaura*	*Aglaura hemistoma*	Yes	No	0	11	0	0	0
Myxozoa	Bivalvulida	Myxidiidae			Yes	No	0	0	0	0	1
Scyphozoa	Semaestomeae	Pelagiidae	*Chrysaora*		Yes	Yes	0	1	0	1	1

Table S3.6: Number of Echinodermata detections in Rottnest Island zooplankton samples by each assay.

Class	Order	Family	Genus	Species	In Australia (ALA, 2016)	Rottnest (ALA, 2016)	Copepod 3	Cnidaria	Crust	Mollusca	Copepod 2	18S
Asteroidea	Velatida				Yes	Yes	0	1	0	0	0	0
Crinoidea	Comatulida				Yes	Yes	0	2	0	0	0	0
		Antedonidae			Yes	Yes	0	0	0	2	0	0
Echinoidea - Echinacea					Yes	Yes	0	0	0	0	0	1
Echinodea	Camarodonta	Echinometridae	*Echinometra*	*Echinometra mathaei*	Yes	Yes	0	2	0	2	0	0
		Temnopleuridae			Yes	Yes	0	0	0	0	2	1
			Temnopleurus	*Temnopleurus michaelseni*	Yes	Yes	10	12	0	5	5	0
	Clypeasteroida				Yes	Yes	0	3	0	0	0	0
	Spatangoida				Yes	Yes	0	8	0	2	0	0
		Loveniidae			Yes	Yes	0	1	0	0	0	0
Ophiuroidea	Ophiurida				Yes	Yes	0	2	0	0	0	1
		Amphiuridae	*Amphipholis*		Yes	Yes	0	0	2	0	0	0
		Ophionereididae	*Ophionereis*	*Ophionereis schayeri*	Yes	Yes	0	2	0	0	0	0

Table S3.7: Number of other Animalia taxa detections in Rottnest Island zooplankton samples by each assay.

Phyla	Class	Order	Family	Genus	Species	In Australia (ALA, 2016)	Rottnest (ALA, 2016)	Copepod 3	Cnidaria	Crust	Mollusca	Copepod 2	Copepod 1	18S
Annelida	Polychaeta	Echiuroidea	Bonelliidae	*Bonellia*		Yes	Yes	0	0	0	1	0	0	0
		Phyllodocida				Yes	Yes	1	2	0	3	0	0	1
		Spionida	Spionidae			Yes	Yes	0	0	0	0	0	0	2
Bryozoa	Gymnolaemata	Cheilostomata	Candidae	*Tricellaria (ALA)*		Yes	No	0	0	1	0	0	0	0
			Membraniporidae	*Biflustra*		Yes	Yes	0	0	0	2	0	0	0
			Watersiporidae	*Watersipora*	*Watersipora cucullata*	Yes	No	0	0	0	2	0	0	0
		Ctenostomatida	Flustrellidridae	*Flustrellidra*	*Flustrellidra armata*	No	No	1	0	0	0	1	2	0
Chaetognatha	Sagittoidea	Aphragmophora	Sagittidae			Yes	Yes	0	1	0	0	0	0	7
				Aidanosagitta	*Aidanosagitta neglecta*	Yes	Yes	0	0	0	0	0	0	2
				Sagitta		Yes	Yes	1	0	0	2	0	2	0
					Sagitta bipunctata	Yes	Yes	0	0	0	0	0	2	0
Ctenophora	Nuda	Berioda	Beroidae	*Beroe*		Yes	No	0	0	0	0	0	0	1
Porifera	Demospongiae					Yes	Yes	0	1	0	0	0	0	0
		Axinellida	Axinellidae			Yes	Yes	0	1	0	0	0	0	0
		Bubarida	Dictyonellidae	*Acanthella*		Yes	Yes	0	1	0	0	0	0	0
		Clionaida	Clionaidae	*Cliona*	*Cliona jullieni*	No	No	0	0	0	3	0	0	0
		Dendroceratida	Darwinellidae	*Dendrilla*		Yes	Yes	1	0	0	0	0	0	0
		Poecilosclerida				Yes	Yes	1	1	0	1	0	0	1
			Hymedesmiidae	*Phorbas*		Yes	Yes	0	0	0	1	0	0	0
			Microcionidae	*Clathria*		Yes	Yes	0	0	0	1	0	0	0
				Ophlitaspongia	*Ophlitaspongia papilla*	No	No	0	0	0	1	0	0	0
		Tetractinellida	Ancorinidae	*Stelletta*		Yes	Yes	0	1	0	0	0	0	0
				Stellettinopsis	*Stellettinopsis megastylifera*	No	No	0	1	0	0	0	0	0
			Geodiinae	*Geodia*		Yes	Yes	0	1	0	0	0	0	0
Nemertea	Anopla	Paleonemertea				No	No	0	0	0	0	0	1	0
			Lineidae			Yes	No	3	0	0	0	0	0	0
	Enopla	Monostilifera	Amphiporidae			Yes	No	1	0	0	0	0	0	0
		Polystilifera	Paradrepanophoridae			No	No	0	2	0	0	0	0	0

Phyla	Class	Order	Family	Genus	Species	In Australia (ALA, 2016)	Rottnest (ALA, 2016)	Copepod 3	Cnidaria	Crust	Mollusca	Copepod 2	Copepod 1	18S
	Paleonemertea					No	No	0	0	0	0	0	1	0
			Cephalothricidae	*Cephalothrix*	*Cephalothrix filiformis*	No	No	0	0	0	0	0	0	1
Platyhelminthes	Rhabditophora	Polycladida				Yes	Yes	0	8	0	1	0	0	0
	Trematoda	Plagiorchiida	Didymozoidae	*Didymozoon*		No	No	0	0	0	0	0	0	1
Sipuncula	Phascolosomatidea	Phascolosomatida	Phascolosomatidae	*Apionsoma*	*Apionsoma (Apionsoma) misakianum*	Yes	No	0	0	0	0	0	0	1

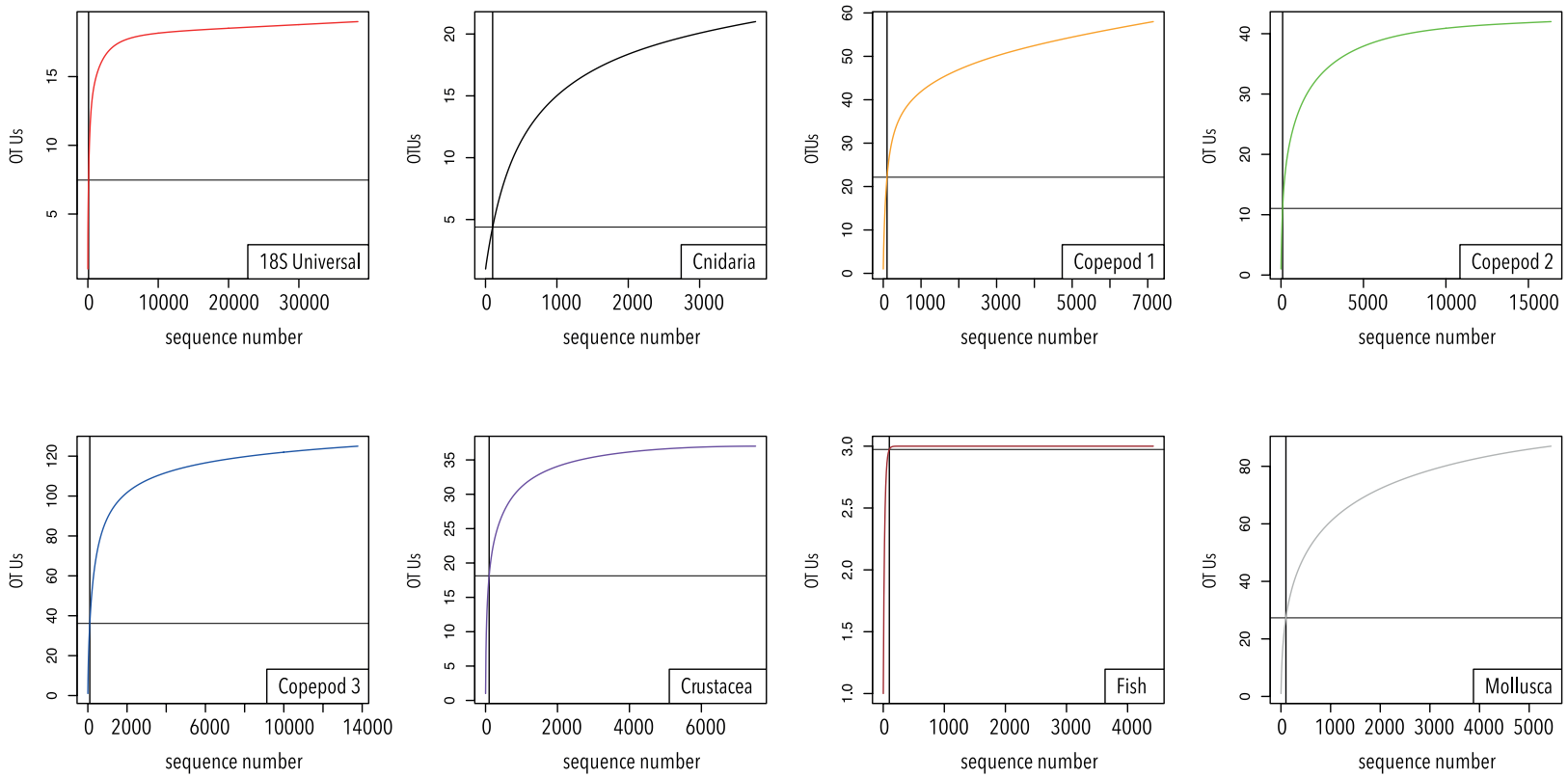


Figure S3.1: Example of typical rarefaction curves. These were produced, using all eight assays, from a single sample taken on May 23 2012

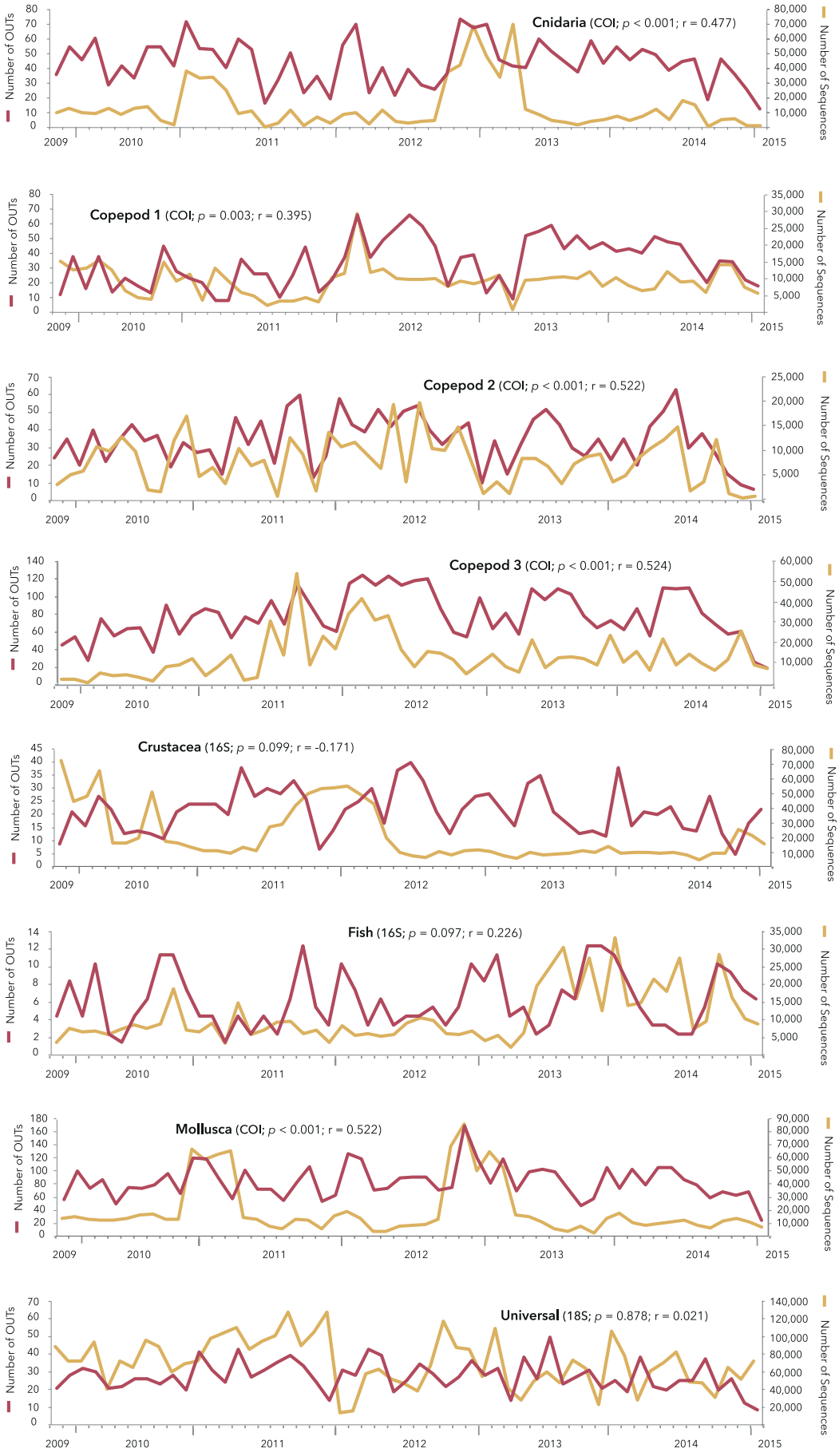

Cnidaria (COI; p < 0.001; r = 0.477)
Copepod 1 (COI; p = 0.003; r = 0.395)
Copepod 2 (COI; p < 0.001; r = 0.522)
Copepod 3 (COI; p < 0.001; r = 0.524)
Crustacea (16S; p = 0.099; r = -0.171)
Fish (16S; p = 0.097; r = 0.226)
Mollusca (COI; p < 0.001; r = 0.522)
Universal (18S; p = 0.878; r = 0.021)
Number of OUTs
Number of Sequences
2009
2010
2011
2012
2013
2014
2015

Figure S3.2: Number of sequences (beige - right axis) and number of OTUs (red - left axis), per sample, for each assay. The assays showed a range of correlations between sequencing depth and the number of OTUs produced. No correlation was detected in the 18S and Crustacea 16S assays, while the Fish 16S showed a weak but non-significant correlation. The COI assays all produced moderate correlations.

Table S3.8: Pairwise analysis of seasonal OTU richness & assemblage, t statistics included for significant results (t)—PERMANOVA+ (Anderson et al. 2008)

Assay	OTU diversity test	Summer Spring	Summer Winter	Summer Autumn	Spring Winter	Spring Autumn	Winter Autumn
Cnidaria	Richness	-	-	-	-	-	-
	Assemblage	* HB t=1.34	*** HB t=2.02	** HB t=1.45	*** HB t=1.62	*** HB t=1.55	** HB t=1.40
Copepod 1	Richness	-	-	-	-	-	-
	Assemblage	* t=1.28	*** HB t=1.85	*** HB t= 1.69	** HB t=1.61	*** HB t=1.71	-
Copepod 2	Richness	-	* t=2.48	-	-	-	-
	Assemblage	-	*** HB t=2.10	-	*** HB t=1.95	** HB t=1.61	-
Copepod 3	Richness	-	-	-	-	-	-
	Assemblage	* t=1.35	*** HB t=2.13	* HB t=1.53	*** HB t=2.12	*** HB t=1.90	-
Crustacea	Richness	* t=2.13	-	-	* =1.76	-	-
	Assemblage	** HB t=1.49	*** HB t=1.76	* HB t=1.40	** HB t=1.54	*** HB t=1.60	* HB t=1.36
Fish	Richness	-	*** HB t =5.03	*** HB t= 3.89	*** HB t= 4.18	** HB t= 3.25	-
	Assemblage	** HB t =1.61	*** HB t =2.20	** HB t =1.73	*** HB t= 2.43	*** HB t =2.23	*** HB t=2.02
Mollusca	Richness	-	-	-	-	-	-
	Assemblage	* HB t=1.31	*** HB t=2.37	** HB t=1.55	*** HB t=1.93	*** HB t=1.55	*** HB t=1.73
Universal	Richness	-	-	-	-	-	-
	Assemblage	* t=1.41	*** HB t=1.87	* t=1.54	** HB t=1.59	* t=1.43	* t=1.40

Where *** is $p \leq .001$, ** is $p \leq .01$, * is $p \leq .05$, HB is significant with Holm-Bonferroni correction & - is no significant changes

Table S3.9: Indicator species analysis for seasonal variation (*Indval*—Roberts, 2016)

Season	Assay	OTU	Taxa	Indicator value	*p* value
Spring	Fish	6	Labridae (two – 96%)	0.6400	0.001
Spring	Universal	37	Hexanauplia	0.4139	0.001
Spring	Copepod 3	125	Perciformes	0.3483	0.004
Spring	Cnidaria	152	Plantae	0.3217	0.007
Spring	Copepod 3	242	*Clausocalanus pergens* (v)	0.3195	0.017
Spring	Fish	12	*Austrolabrus maculatus*	0.3176	0.012
Spring	Copepod 3	186	*Bestiolina sp*	0.3150	0.020
Spring	Mollusca	43	Mullidae	0.3148	0.008
Spring	Copepod 2	40	Hexanauplia	0.3006	0.014
Spring	Copepod 3	115	Paracalanidae	0.2950	0.011
Spring	Cnidaria	18	Arthropoda	0.2948	0.045
Spring	Fish	11	*Upeneichthys stotti*	0.2904	0.017
Spring	Copepod 3	2	*Bestiolina sp.*	0.2833	0.025
Spring	Copepod 3	166	*Bestiolina sp.*	0.2816	0.017
Spring	Copepod 1	82	*Dicathais orbita* (v)	0.2708	0.011
Spring	Copepod 3	303	Labridae	0.2667	0.005
Spring	Cnidaria	38	Leptothecata	0.2667	0.010
Spring	Copepod 3	128	*Evadne spinifera* (v)	0.2659	0.038
Spring	Cnidaria	37	Echinoidea	0.2430	0.043
Spring	Mollusca	202	Animalia	0.2299	0.022
Spring	Mollusca	251	Anopla	0.2286	0.033
Spring	Copepod 1	41	Gastropoda	0.2133	0.026
Spring	Crustacea	36	Arthropoda	0.2083	0.033
Spring	Mollusca	245	*Centropages violaceus* (v)	0.2000	0.048
Spring	Cnidaria	214	Phyllodocida.	0.2000	0.050
Spring	Cnidaria	69	Cnidaria	0.1778	0.047
Summer	Copepod 3	22	*Calcinus dapsiles*	0.6661	0.001
Summer	Crustacea	16	*Calcinus dapsiles*	0.4952	0.001
Summer	Cnidaria	13	*Evadne spinifera* (v)	0.4535	0.001
Summer	Mollusca	3	*Evadne spinifera* (v)	0.4258	0.001
Summer	Copepod 3	296	Calanidae	0.4000	0.002
Summer	Cnidaria	17	*Etrumeus jacksoniensis*	0.3786	0.001
Summer	Mollusca	93	Arthropoda	0.3752	0.004
Summer	Cnidaria	34	Chlorophyta	0.3663	0.004
Summer	Mollusca	88	Malacostraca	0.3661	0.003
Summer	Mollusca	109	Malacostraca	0.3607	0.002
Summer	Cnidaria	91	*Chromodoris sp.*	0.3510	0.004
Summer	Copepod 1	109	*Triconia sp.*	0.3437	0.005
Summer	Mollusca	263	*Chromodoris striatella* (v)	0.3355	0.006
Summer	Fish	37	*Chromis sp.*	0.3333	0.004
Summer	Cnidaria	15	Arthropoda	0.3298	0.016
Summer	Cnidaria	10	Animalia	0.3283	0.006
Summer	Mollusca	257	Animalia	0.3242	0.009
Summer	Copepod 3	194	Arthropoda	0.3169	0.006
Summer	Copepod 1	34	*Triconia sp.*	0.3169	0.007
Summer	Cnidaria	14	Hydrozoa	0.3029	0.033
Summer	Mollusca	179	Hexanauplia	0.2889	0.009

Season	Assay	OTU	Taxa	Indicator value	*p* value
Summer	Mollusca	184	Hydrozoa	0.2877	0.024
Summer	Mollusca	247	*Trachurus sp.* (Two at 100% (v)).	0.2778	0.015
Summer	Mollusca	97	Podonidae	0.2751	0.036
Summer	Mollusca	106	Rhodophyta	0.2716	0.019
Summer	Mollusca	5	*Cliona jullieni*	0.2708	0.012
Summer	Cnidaria	86	Alveolata	0.2683	0.033
Summer	Mollusca	54	Biemnidae (Two at 100% (v))	0.2667	0.012
Summer	Mollusca	343	Arthropoda	0.2667	0.017
Summer	Mollusca	292	Arthropoda	0.2595	0.016
Summer	Universal	15	Eukaryota	0.2579	0.015
Summer	Mollusca	137	Calanoida	0.2553	0.037
Summer	Mollusca	267	Malacostraca	0.2537	0.016
Summer	Crustacea	45	Animalia	0.2537	0.026
Summer	Cnidaria	135	Chlorophyta	0.2537	0.031
Summer	Cnidaria	71	Arthropoda	0.2483	0.042
Summer	Copepod 3	247	*Actinaria* sp. (v)	0.2328	0.022
Summer	Mollusca	84	Animalia	0.2307	0.031
Summer	Mollusca	281	Arthropoda	0.2307	0.037
Summer	Mollusca	20	Sapphirinidae	0.2299	0.028
Summer	Copepod 3	176	*Chromis sp.*	0.2133	0.028
Summer	Cnidaria	46	*Pleurobranchus hilli*	0.2133	0.029
Summer	Mollusca	108	Annelida	0.2133	0.031
Summer	Copepod 3	59	*Chromis notata*	0.2070	0.045
Summer	Cnidaria	167	Gastropoda	0.2044	0.039
Summer	Fish	13	*Chromis notata*	0.2032	0.044
Summer	Universal	87	Chromista	0.1778	0.045
Autumn	Mollusca	85	*Farranula gibbula* (v)	0.3858	0.002
Autumn	Copepod 3	55	*Flaccisagitta enflata*	0.3844	0.001
Autumn	Copepod 3	90	*Centropages orsinii*	0.3757	0.002
Autumn	Fish	3	*Engraulis sp.* (Three at 100%)	0.3630	0.003
Autumn	Universal	79	Sagittidae	0.3611	0.003
Autumn	Cnidaria	30	*Temnopleurus michaelseni*	0.3528	0.010
Autumn	Crustacea	63	Arthropoda	0.35	0.007
Autumn	Crustacea	34	Animalia	0.3362	0.015
Autumn	Copepod 1	122	*Undinula vulgaris* (v)	0.3326	0.005
Autumn	Copepod 1	37	*Clausocalanus minor*	0.3313	0.005
Autumn	Cnidaria	61	Prasinophyceae	0.3297	0.034
Autumn	Copepod 3	293	Bivalvia	0.3278	0.002
Autumn	Crustacea	30	Animalia	0.3278	0.003
Autumn	Cnidaria	184	Ophiuroidea	0.3278	0.005
Autumn	Crustacea	35	Animalia	0.3278	0.007
Autumn	Universal	23	Eukaryota	0.3248	0.020
Autumn	Copepod 3	122	*Calocalanus pavo (v)*	0.3159	0.016
Autumn	Copepod 3	290	Malacostraca	0.3077	0.005
Autumn	Copepod 3	104	Sagittidae	0.3074	0.021
Autumn	Mollusca	33	Decapoda	0.3074	0.040
Autumn	Copepod 3	216	Copepoda	0.3036	0.013
Autumn	Cnidaria	117	Animalia	0.3033	0.019
Autumn	Copepod 1	97	Caenogastropoda	0.2984	0.014

Season	Assay	OTU	Taxa	Indicator value	*p* value
Autumn	Universal	42	Sargassaceae (Many at 100%)	0.2984	0.022
Autumn	Copepod 1	125	*Acrocalanus gracilis* (v)	0.2979	0.032
Autumn	Copepod 3	139	*Paracalanus sp.* (Two at 100%)	0.2838	0.020
Autumn	Copepod 3	266	Ophiurida	0.2797	0.014
Autumn	Crustacea	74	Animalia	0.2767	0.010
Autumn	Copepod 3	80	*Temnopleurus michaelseni*	0.2751	0.044
Autumn	Mollusca	45	Animalia	0.2740	0.038
Autumn	Mollusca	28	Animalia	0.2705	0.026
Autumn	Cnidaria	23	Hydrozoa	0.2530	0.022
Autumn	Copepod 3	297	Euralida	0.2530	0.025
Autumn	Copepod 2	66	*Centropages orsinii*	0.2529	0.012
Autumn	Crustacea	12	Eukaryota	0.2510	0.048
Autumn	Mollusca	259	Annelida	0.2473	0.034
Autumn	Copepod 2	80	*Acrocalanus sp.*	0.2412	0.047
Autumn	Copepod 3	188	*Sagitta sp.*	0.2331	0.034
Autumn	Crustacea	9	*Menaethius sp.*	0.2069	0.042
Winter	Mollusca	68	*Canthocalanus pauper*	0.4987	0.001
Winter	Copepod 3	110	*Centropages furcatus*	0.4952	0.001
Winter	Mollusca	158	Poecilostomatoida	0.4710	0.001
Winter	Copepod 2	19	Hexanauplia	0.4646	0.001
Winter	Mollusca	78	Calanoida	0.4447	0.003
Winter	Cnidaria	90	Gastropoda	0.4288	0.002
Winter	Mollusca	174	Arthropoda	0.4183	0.004
Winter	Mollusca	38	*Eucalanus pseudoattenuatus* (v)	0.4148	0.003
Winter	Copepod 1	11	*Eucalanus pseudattenuatus (v)*	0.4088	0.002
Winter	Mollusca	180	Arthropoda	0.4050	0.001
Winter	Copepod 2	18	Hexanauplia	0.3985	0.004
Winter	Copepod 2	46	Crustacea	0.3976	0.002
Winter	Mollusca	47	*Subeucalanus pileatus* (v)	0.3965	0.005
Winter	Copepod 2	55	*Paracalanus sp.* (Two at 100%(v))	0.3908	0.005
Winter	Universal	91	Pycnococcaceae (Many at 100%)	0.3852	0.003
Winter	Copepod 3	93	*Paracalanus sp.* (Two at 100%(v))	0.3824	0.002
Winter	Mollusca	107	Calanoida	0.3779	0.007
Winter	Copepod 3	121	Sagittidae	0.3777	0.008
Winter	Copepod 3	83	*Acrocalanus gracilis*	0.3692	0.007
Winter	Mollusca	130	*Ecklonia radiata*	0.3685	0.005
Winter	Universal	52	*Leptochela sp*	0.3592	0.001
Winter	Copepod 3	138	Calanoida	0.3552	0.005
Winter	Copepod 2	5	*Lucicutia flavicornis* (v)	0.3540	0.009
Winter	Copepod 2	44	*Eucalanus pseudattenuatus* (v)	0.3504	0.014
Winter	Copepod 3	73	Calanoida	0.3486	0.012
Winter	Copepod 3	58	*Flaccisagitta enflata*	0.3394	0.021
Winter	Mollusca	144	*Paracalanus sp.* (Two at 100% (v))	0.3380	0.033
Winter	Copepod 2	32	*Paracalanus sp.*	0.3373	0.014
Winter	Mollusca	79	*Calocalanus pavo* (v)	0.3364	0.013
Winter	Copepod 3	107	Calanoida	0.3346	0.006
Winter	Cnidaria	12	*Bassia bassensis*	0.3283	0.008
Winter	Copepod 2	7	*Delibus sp.*	0.3283	0.043
Winter	Copepod 3	37	*Undinula vulgaris* (v)	0.3267	0.024

Season	Assay	OTU	Taxa	Indicator value	*p* value
Winter	Copepod 2	35	Paracalanidae	0.3267	0.026
Winter	Mollusca	110	Animalia	0.3251	0.025
Winter	Copepod 3	14	*Cosmocalanus darwinii*	0.3223	0.047
Winter	Copepod 2	16	*Clausocalanus minor*	0.3221	0.048
Winter	Mollusca	116	Arthropoda	0.3209	0.008
Winter	Copepod 3	85	*Canthocalanus pauper*	0.3205	0.029
Winter	Cnidaria	45	Animalia	0.3183	0.022
Winter	Copepod 3	200	Arthropoda	0.3176	0.007
Winter	Copepod 3	215	*Euchaeta concinna* (v)	0.3176	0.007
Winter	Copepod 3	52	*Eucalanus pseudattenuatus* (v)	0.3168	0.030
Winter	Copepod 3	274	*Subeucalanus pileatus* (v)	0.3157	0.007
Winter	Copepod 2	95	*Scolecithricella longispinosa* (v)	0.3099	0.006
Winter	Copepod 3	64	*Sagitta sp.*	0.3074	0.040
Winter	Copepod 3	78	*Flaccisagitta enflata*	0.3074	0.048
Winter	Copepod 3	79	*Scolecithrix danae* (v)	0.3070	0.034
Winter	Mollusca	220	Calanoida (Two at 98% (v))	0.3062	0.024
Winter	Copepod 1	69	*Paracalanus sp.*	0.3062	0.024
Winter	Copepod 3	145	Arthropoda	0.3011	0.046
Winter	Copepod 3	50	*Paracalanus aculeatus*	0.3011	0.048
Winter	Cnidaria	96	Animalia	0.2995	0.045
Winter	Copepod 1	12	Arthropoda	0.2953	0.048
Winter	Cnidaria	112	*Pontellina plumata* (v)	0.2928	0.007
Winter	Mollusca	51	*Lucicutia flavicornis* (v)	0.2923	0.039
Winter	Mollusca	50	Hexanauplia	0.2913	0.028
Winter	Copepod 3	111	*Lucifer intermedius*	0.2893	0.015
Winter	Copepod 3	189	*Sagitta sp.*	0.2888	0.018
Winter	Copepod 3	217	Arthropoda	0.2778	0.009
Winter	Copepod 2	53	Calanoida	0.2778	0.009
Winter	Copepod 2	84	Palaeonemertea	0.2778	0.010
Winter	Copepod 3	101	*Cosmocalanus sp.*	0.2756	0.026
Winter	Copepod 3	244	Arthropoda	0.2725	0.018
Winter	Copepod 3	282	Annelida	0.2708	0.011
Winter	Mollusca	17	*Lucifer sp.* (v)	0.2686	0.020
Winter	Mollusca	132	Animalia	0.2678	0.033
Winter	Crustacea	31	Animalia	0.2657	0.023
Winter	Mollusca	171	Animalia	0.2657	0.046
Winter	Copepod 3	208	Arthropoda	0.2586	0.023
Winter	Copepod 3	196	*Flaccisagitta enflata*	0.2574	0.045
Winter	Copepod 1	76	*Calocalanus pavo* (v)	0.2574	0.047
Winter	Copepod 1	32	*Animalia*	0.2500	0.003
Winter	Copepod 3	113	Valvatida	0.2500	0.006
Winter	Crust 16S	125	Animalia	0.2500	0.006
Winter	Copepod 3	65	Arthropoda	0.2500	0.012
Winter	Copepod 2	31	Hexanauplia	0.2481	0.047
Winter	Copepod 1	148	*Calanoida*	0.2397	0.035
Winter	Crustacea	111	Malacostraca	0.2381	0.017
Winter	Copepod 1	28	Calanidae	0.2327	0.049
Winter	Cnidaria	89	Asteroidea	0.2126	0.040
Winter	Mollusca	321	Arthropoda	0.1912	0.037

Season	Assay	OTU	Taxa	Indicator value	*p* value
Winter	Copepod 3	261	Anthoathecata	0.1912	0.039
Winter	Cnidaria	148	*Sagitta sp.*	0.1912	0.049
Winter	Crustacea	60	Arthropoda	0.1667	0.036
Winter	Copepod 3	182	Ophiurida	0.1667	0.044
Winter	Cnidaria	238	Cidaridae	0.1667	0.045
Winter	Copepod 2	72	Animalia	0.1667	0.049
Winter	Copepod 1	154	*Arthropoda*	0.1667	0.050

(v) Matched to vouchered specimen sequence

Table S3.10 Pairwise analysis of yearly OTU richness & assemblage, t statistics included for significant results (t)—PERMANOVA+ (Anderson et al. 2008)

Assay	OTU diversity test	2010 2011	2010 2012	2010 2013	2010 2014	2011 2012	2011 2013	2011 2014	2012 2013	2012 2014	2013 2014
Cnidaria	Richness	-	-	-	-	-	-	-	-	-	-
	Assemblage	-	*** HB t=1.63	*** HB t=1.81	*** HB t=2.01	* t=1.33	** HB t=1.49	** HB t=1.51	-	-	-
Copepod 1	Richness	-	* t=2.89	-	* t=2.99	** HB t=3.81	* t=2.67	*** HB t=4.10	-	-	-
	Assemblage	* t=1.38	*** HB t=1.96	*** HB t=1.85	*** HB t=2.33	** HB t=1.73	** HB t=1.57	*** HB t=2.07	-	* t=1.28	* t =1.32
Copepod 2	Richness	-	* t=2.73	-	-	-	-	-	-	-	-
	Assemblage	* t=1.37	** t=1.51	-	-	* t=1.45	-	* t=1.37	-	* t=1.34	-
Copepod 3	Richness	* t=2.50	** HB t= 3.61	** t=3.04	* t=2.29	* t=2.23	-	-	-	-	-
	Assemblage	* t=1.32	** HB t=1.71	** HB t=1.48	* t=1.40	** t=1.49	* t=1.37	* t=1.47	-	-	-
Crustacea	Richness	* t=2.58	* t= 2.14	-	-	-	-	-	-	-	-
	Assemblage	-	-	-	-	-	-	-	-	** t=1.45	-
Fish	Richness	-	-	-	-	-	-	-	-	-	-
	Assemblage	-	-	-	-	-	-	-	-	-	-
Mollusca	Richness	-	-	-	-	-	-	-	-	-	-
	Assemblage	* t=1.30	*** HB t=1.73	*** HB t=1.57	** HB t=1.65	** HB t=1.48	** HB t =1.43	** HB t =1.56	-	-	-
Universal	Richness	-	-	-	-	-	-	-	-	-	-
	Assemblage	-	* t=1.34	** HB t=1.46	*** HB t= 1.67	-	-	** t =1.86	-	-	-

Where *** is $p \leq .001$, ** is $p \leq .01$, * is $p \leq .05$, HB is significant with Holm-Bonferroni correction & - is no significant changes

Table S3.11: Indicator species analysis for yearly variation (*Indval*—Roberts, 2016)

Year	Assay	OTU	Taxa	Indicator value	*p* value
2010	Cnidaria	165	Pythiales	0.6667	0.001
2010	Cnidaria	175	Animalia	0.4540	0.001
2010	Mollusca	216	Arthropoda	0.4444	0.001
2010	Copepod 2	11	Arthropoda	0.4444	0.001
2010	Copepod 1	57	Arthropoda	0.4123	0.001
2010	Mollusca	36	*Creseis sp.*	0.4085	0.002
2010	Mollusca	333	*Oncaea venusta typica*	0.3612	0.008
2010	Mollusca	137	Calanoida	0.3341	0.004
2010	Crustacea	24	Xanthidae	0.3333	0.003
2010	Cnidaria	21	Hydrozoa	0.3325	0.012
2010	Copepod 1	74	*Paracalanus Indicus*	0.3320	0.014
2010	Mollusca	118	Drepanophoridae	0.3099	0.027
2010	Cnidaria	95	Oithonidae	0.3045	0.016
2010	Cnidaria	54	*Acartia negligens*	0.2941	0.035
2010	Cnidaria	24	*Aglaura hemistoma*	0.2911	0.022
2010	Cnidaria	22	*Penilia avirostris*	0.2910	0.050
2010	Cnidaria	72	Thalassiosirales	0.2736	0.030
2010	Fish	9	Perciformes	0.2500	0.023
2010	Copepod 3	285	Calanoida	0.2222	0.026
2010	Copepod 3	280	Hexanauplia	0.2222	0.031
2010	Mollusca	252	Polychaeta	0.2222	0.032
2010	Copepod 1	110	Gastropoda	0.2222	0.032
2010	Copepod 1	133	Gastropoda	0.2222	0.032
2010	Universal	78	*Centropages sp.*	0.2222	0.036
2011	Mollusca	17	*Lucifer sp.*	0.4760	0.002
2011	Copepod 3	111	*Lucifer intermedius*	0.3969	0.002
2011	Crustacea	31	Animalia	0.3645	0.004
2011	Cnidaria	105	Hydrozoa	0.3636	0.010
2011	Mollusca	280	*Undinula vulgaris*	0.3240	0.005
2011	Universal	18	Decapoda	0.2978	0.018
2011	Cnidaria	45	Animalia	0.2968	0.026
2011	Copepod 2	107	Mollusca	0.2909	0.016
2011	Crustacea	26	*Thalamita admete*	0.2852	0.012
2011	Universal	33	Heterobranchia	0.2727	0.025
2011	Copepod 3	271	Sagittoidea	0.2727	0.029
2011	Cnidaria	125	Plantae	0.2680	0.045
2011	Universal	38	Palaemonoidea	0.2630	0.032
2011	Universal	71	*Temora sp.*	0.2625	0.050
2011	Copepod 3	85	*Canthocalanus pauper*	0.2583	0.045
2011	Cnidaria	98	Animalia	0.2528	0.049
2011	Universal	61	Syndiniales	0.2424	0.024
2011	Copepod 3	300	*Clausocalanus sp.*	0.2385	0.039
2011	Universal	87	Chromista	0.2385	0.044
2011	Mollusca	8	*Candacia bradyi*	0.2286	0.007
2011	Mollusca	14	*Delibus sp.*	0.2234	0.014
2012	Mollusca	99	Arthropoda	0.4069	0.001
2012	Copepod 3	49	*Lucifer intermedius*	0.4040	0.001

Year	Assay	OTU	Taxa	Indicator value	*p* value
2012	Copepod 2	71	Calanoida	0.3997	0.001
2012	Copepod 3	154	*Paracalanus sp.*	0.3707	0.006
2012	Mollusca	75	Arthropoda	0.3378	0.003
2012	Copepod 3	181	Mollusca	0.3349	0.007
2012	Mollusca	96	Animalia	0.3344	0.009
2012	Cnidaria	16	Chlorophyta	0.3235	0.001
2012	Copepod 3	158	*Calocalanus sp.*	0.3228	0.006
2012	Mollusca	192	Calanoida	0.3223	0.011
2012	Mollusca	4	Eukaryota	0.3152	0.001
2012	Copepod 1	22	Paracalanidae	0.3091	0.014
2012	Mollusca	73	Malacostraca	0.3075	0.017
2012	Copepod 2	61	Calanoida	0.3046	0.022
2012	Copepod 3	86	*Calocalanus styliremis*	0.2972	0.020
2012	Copepod 3	89	Arthropoda	0.2968	0.022
2012	Copepod 3	74	*Lirabuccinum sp.*	0.2911	0.016
2012	Mollusca	134	Gastropoda	0.2909	0.021
2012	Copepod 1	36	Arthropoda	0.2868	0.013
2012	Crustacea	102	Eukaryota	0.2852	0.012
2012	Copepod 3	202	Arthropoda	0.2847	0.038
2012	Copepod 1	81	Arthropoda	0.2840	0.016
2012	Copepod 1	4	*Oncaea waldemari*	0.2758	0.026
2012	Copepod 3	301	Potamididae	0.2731	0.022
2012	Copepod 3	321	Arthropoda	0.2727	0.030
2012	Copepod 3	20	Arthropoda	0.2727	0.032
2012	Copepod 3	237	*Paracalanus nanus*	0.2683	0.018
2012	Cnidaria	228	Animalia	0.2676	0.050
2012	Copepod 3	33	Calanoida	0.2653	0.001
2012	Copepod 3	63	*Ditrichocorycaeus sp.*	0.2565	0.018
2012	Copepod 2	49	*Paracalanus sp.*	0.2562	0.038
2012	Copepod 2	98	Mollusca	0.2525	0.025
2012	Crustacea	97	Animalia	0.2438	0.019
2012	Copepod 3	250	Calanoida	0.2424	0.028
2012	Copepod 1	68	Arthropoda	0.2424	0.032
2012	Copepod 3	293	Bivalvia	0.2385	0.041
2012	Copepod 3	332	*Paracalanus sp.*	0.2285	0.050
2013	Cnidaria	5	Prasinophyceae	0.4231	0.001
2013	Copepod 1	56	Arthropoda	0.4000	0.003
2013	Copepod 1	19	Arthropoda	0.3143	0.013
2013	Mollusca	259	Annelida	0.3088	0.007
2013	Mollusca	63	Mollusca	0.3046	0.023
2013	Universal	13	Chlorophyta	0.3037	0.002
2013	Cnidaria	154	Gastropoda	0.3000	0.012
2013	Cnidaria	38	Leptothecata	0.3000	0.013
2013	Cnidaria	131	Animalia	0.3000	0.014
2013	Cnidaria	30	*Temnopleurus michaelseni*	0.2969	0.031
2013	Copepod 3	25	*Labidocera minuta*	0.2903	0.016
2013	Copepod 2	1	Mollusca	0.2886	0.029
2013	Mollusca	30	*Cacozeliana granarium*	0.2843	0.045
2013	Copepod 3	43	Paracalanidae	0.2829	0.035

Year	Assay	OTU	Taxa	Indicator value	*p* value
2013	Cnidaria	50	*Sagitta sp.*	0.2826	0.035
2013	Cnidaria	199	Nudibranchia	0.2750	0.010
2013	Mollusca	5	*Cliona jullieni*	0.2750	0.020
2013	Copepod 3	26	Arthropoda	0.2694	0.043
2013	Cnidaria	99	Nudibranchia	0.2676	0.036
2013	Universal	79	Sagittidae	0.2672	0.031
2013	Universal	41	Cypridinidae	0.2619	0.018
2013	Mollusca	188	Pyramimonadophyceae	0.2571	0.020
2013	Cnidaria	11	Hydrozoa	0.2565	0.021
2013	Copepod 1	84	Arthropoda	0.2538	0.039
2013	Mollusca	309	Mollusca	0.2378	0.031
2013	Copepod 1	106	Animalia	0.2302	0.039
2013	Cnidaria	242	Arthropoda	0.2302	0.042
2014	Copepod 1	44	*Triconia sp.*	0.4046	0.002
2014	Copepod 1	42	Arthropoda	0.3610	0.002
2014	Mollusca	64	*Oncaea sp.*	0.3146	0.007
2014	Copepod 1	54	Arthropoda	0.3006	0.008
2014	Cnidaria	104	Chlorophyta	0.2955	0.011
2014	Copepod 3	66	*Ctenocalanus vanus*	0.2926	0.007
2014	Copepod 1	62	*Oncaea mediterranea* (v)	0.2874	0.022
2014	Copepod 3	217	Arthropoda	0.2852	0.014
2014	Copepod 1	167	Arthropoda	0.2852	0.018
2014	Copepod 3	28	Muricidae	0.2839	0.010
2014	Copepod 1	31	*Ataxocerithium*	0.2780	0.045
2014	Mollusca	27	Calanoida	0.2772	0.011
2014	Crustacea	132	Eukaryota	0.2727	0.024
2014	Crustacea	20	Eukaryota	0.2727	0.034
2014	Copepod 1	32	Animalia	0.2727	0.045
2014	Copepod 1	20	Arthropoda	0.2725	0.029
2014	Mollusca	178	*Paracalanus nanus* (v)	0.2686	0.044
2014	Cnidaria	55	Viridaeplantae	0.2680	0.038
2014	Cnidaria	91	*Chromodoris sp.*	0.2470	0.023
2014	Copepod 1	21	Gastropoda	0.2346	0.039
2014	Copepod 3	39	Calanoida	0.2281	0.034

(v) Matched to vouchered specimen sequence

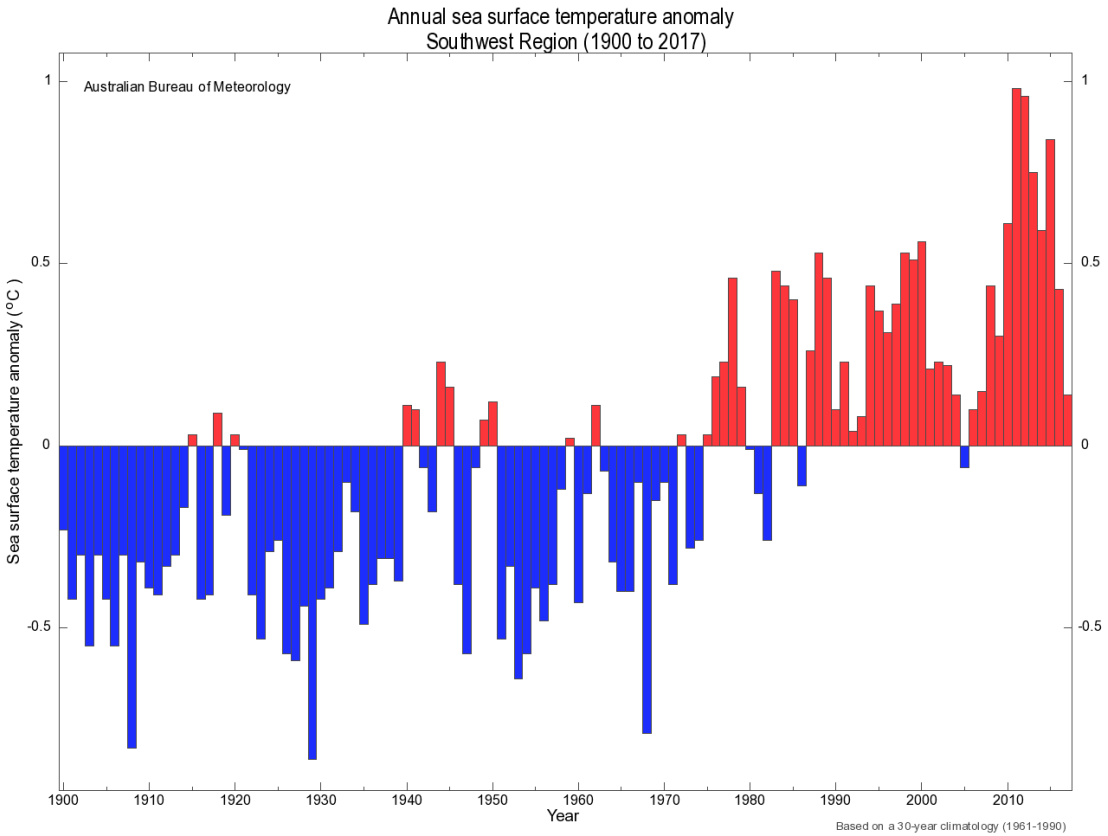


Figure S3.3: Annual sea surface temperature anomalies from 1900-2017. The 2011 and 2012 heatwave events produce the two highest peaks —Extracted from the Bureau of Meteorology time series graphs (accessed December 2018).

Table S3.12: Pairwise analysis of heatwave OTU richness & assemblage between before, during and after the heatwaves, t statistics included for significant results (t)—PERMANOVA+ (Anderson et al. 2008)

Assay	OTU diversity test	Heatwave 1 (5 Months)			Heatwave 2 (17 Months)		
		Before-During	Before-After	During-After	Before-During	Before-After	During-After
Cnidaria	Richness	-	-	-	-	-	-
	Assemblage	* HB t=1.27	*** HB t=1.93	** HB t=1.46	* HB t=1.37	*** HB t=2.12	** HB t =1.48
Copepod 1	Richness	-	* HB t=2.46	** HB t=2.88	-	* HB t=2.71	-
	Assemblage	-	*** HB t= 2.19	* t=1.55	** HB t=1.48	*** HB t =2.39	*** HB t =1.75
Copepod 2	Richness	-	-	-	-	-	-
	Assemblage	-	-	-	-	-	* HB t =1.56
Copepod 3	Richness	-	** HB t=2.84	-	** HB t=3.19	* t=2.34	-
	Assemblage	-	** HB t =1.58	-	** HB t=1.55	* HB t =1.48	* HB t=1.51
Crustacea	Richness	* t=2.32	-	-	** HB t= 2.95	-	-
	Assemblage	-	-	-	-	-	-
Fish	Richness	-	-	-	-	-	-
	Assemblage	-	-	* HB t=1.44	-	-	-
Mollusca	Richness	-	-	-	-	-	-
	Assemblage	-	** HB t=1.64	** HB t=1.54	** HB t=1.43	** HB t=1.62	** HB t=1.47
Universal	Richness	-	-	-	-	-	-
	Assemblage	-	** HB t=1.59	* t=1.41	-	** HB t=1.68	** HB t=1.64

Where *** is $p \leq .001$, ** is $p \leq .01$, * is $p \leq .05$, HB is significant with Holm-Bonferroni correction & - is no significant change

Table S3.13: Indicator species analysis for five-month heatwave variation (*Indval*—Roberts, 2016)

Heatwave	Assay	OTU	Taxa	Indicator value	*p* value
Before	Cnidaria	165	Pythiales	0.7778	0.001
Before	Copepod 1	74	*Paracalanus Indicus*	0.6061	0.002
Before	Copepod 2	14	Calanoida	0.4972	0.023
Before	Copepod 1	57	Arthropoda	0.4855	0.018
Before	Copepod 1	13	Paracalanidae	0.4638	0.015
Before	Universal	23	Alveolata	0.4628	0.030
Before	Cnidaria	87	Chlorophyta	0.4098	0.047
Before	Cnidaria	21	Hydrozoa	0.3984	0.030
Before	Copepod 1	114	Arthropoda	0.3644	0.031
Before	Copepod 2	11	Arthropoda	0.3065	0.036
Before	Mollusca	216	Arthropoda	0.3065	0.037
Before	Cnidaria	168	Plantae	0.2908	0.049
Before	Copepod 3	285	Calanoida	0.2222	0.033
Before	Copepod 1	110	Gastropoda	0.2222	0.033
Before	Copepod 1	133	Gastropoda	0.2222	0.035
Before	Mollusca	252	Polychaeta	0.2222	0.037
During	Mollusca	179	Hexanauplia	0.6502	0.001
During	Copepod 3	41	Actinopterygii	0.6052	0.004
During	Mollusca	201	*Euchaeta longicornis*	0.5715	0.002
During	Cnidaria	125	Plantae	0.5426	0.007
During	Copepod 3	95	Arthropoda	0.5426	0.010
During	Fish	5	*Parapriacanthus elongatus*	0.5256	0.017
During	Mollusca	242	*Euphausia hemigibba*	0.5063	0.004
During	Universal	71	*Temora sp.*	0.5055	0.011
During	Mollusca	87	*Copilia mirabilis* (v)	0.4980	0.019
During	Cnidaria	160	Arthropoda	0.4959	0.018
During	Mollusca	37	*Turritopsis sp.* (Two – 98%)	0.4867	0.019
During	Mollusca	57	Arthropoda	0.4862	0.026
During	Copepod 3	130	*Subeucalanus sp.* (Two – 100%(v))	0.4827	0.024
During	Copepod 3	22	*Calcinus dapsiles*	0.4779	0.025
During	Mollusca	145	*Euphasia recurva* (v)	0.4684	0.011
During	Mollusca	280	*Undinula vulgaris*	0.4671	0.009
During	Cnidaria	158	Corycaeidae	0.4647	0.025
During	Cnidaria	11	Hydrozoa	0.4624	0.011
During	Cnidaria	44	Malacostraca	0.4566	0.041
During	Mollusca	76	*Subeucalanus sp.* (two – 99%)	0.4488	0.033
During	Cnidaria	10	Animalia	0.4413	0.049
During	Mollusca	267	Malacostraca	0.4321	0.029
During	Copepod 3	106	Arthropoda	0.4201	0.050
During	Crustacea	88	Animalia	0.4198	0.025
During	Mollusca	186	Eukaryota	0.4000	0.005
During	Mollusca	197	Gastropoda	0.4000	0.008
During	Mollusca	307	Eukaryota	0.4000	0.008
During	Mollusca	7	Parazoanthidae (Many – 99%)	0.4000	0.008
During	Mollusca	224	Gastropoda	0.4000	0.010
During	Mollusca	209	Arthropoda	0.4000	0.010

Heatwave	Assay	OTU	Taxa	Indicator value	*p* value
During	Mollusca	80	Aglajidae	0.4000	0.011
During	Copepod 3	91	*Euphausia recurva* (v)	0.3813	0.040
During	Universal	33	Heterobranchia	0.3770	0.013
During	Mollusca	15	*Watersipora subovoidea*	0.3770	0.013
During	Cnidaria	203	Animalia	0.3770	0.014
During	Mollusca	278	Calanoida	0.3770	0.014
During	Cnidaria	128	*Subeucalanus mucronatus* (v)	0.3770	0.016
During	Copepod 3	126	*Stylocheiron sp.*	0.3770	0.018
During	Mollusca	152	*Subeucalanus mucronatus* (v)	0.3770	0.021
During	Cnidaria	81	Echinodermata	0.3717	0.050
During	Copepod 3	246	Calanoida	0.3565	0.020
During	Cnidaria	201	Eukaryota	0.3565	0.022
During	Cnidaria	73	Unknown	0.3565	0.037
During	Cnidaria	105	Hydrozoa	0.3565	0.037
During	Copepod 2	107	Mollusca	0.3381	0.027
During	Mollusca	211	Arthropoda	0.3381	0.029
During	Mollusca	108	Annelida	0.3381	0.037
During	Crustacea	13	Animalia	0.3381	0.038
During	Cnidaria	46	*Pleurobranchus hilli*	0.3381	0.040
During	Mollusca	135	Caenogastropoda	0.3381	0.043
During	Universal	59	*Creseis sp.*	0.3381	0.044
During	Mollusca	5	*Cliona sp.*	0.3216	0.037
During	Copepod 1	60	Arthropoda	0.3216	0.037
During	Copepod 3	300	*Clausocalanus sp.*	0.3216	0.040
During	Crustacea	67	Eukaryota	0.3216	0.047
During	Universal	87	Chromista	0.3216	0.050
During	Copepod 3	150	Arthropoda	0.3130	0.019
During	Mollusca	121	Mollusca	0.3130	0.026
During	Copepod 3	264	*Euphausia hemigibba*	0.2988	0.033
During	Mollusca	185	Gastropoda	0.2988	0.048
After	Mollusca	4	Eukaryota	0.8780	0.001
After	Mollusca	64	*Oncaea sp.* (Two – 100%)	0.7073	0.001
After	Cnidaria	16	Chlorophyta	0.6681	0.001
After	Cnidaria	5	Prasinophyceae	0.6585	0.001
After	Copepod 3	28	Muricidae	0.6352	0.001
After	Copepod 1	33	Gastropoda	0.6341	0.002
After	Copepod 1	36	Arthropoda	0.5746	0.002
After	Copepod 1	4	*Oncaea waldemari* (v)	0.5463	0.002
After	Cnidaria	104	Chlorophyta	0.5158	0.018
After	Universal	13	Chlorophyta	0.5131	0.002
After	Mollusca	75	Arthropoda	0.5122	0.014
After	Copepod 3	50	*Paracalanus aculeatus*	0.4920	0.016
After	Copepod 1	30	Neogastropoda	0.4878	0.015
After	Copepod 2	25	*Paracalanus sp. (*Two at 100%)	0.4696	0.034
After	Copepod 3	89	Arthropoda	0.4634	0.026
After	Copepod 1	44	Triconia sp.	0.4634	0.029
After	Universal	14	Chlorellaceae (Many at 99%)	0.4445	0.036
After	Mollusca	45	Animalia	0.4390	0.038
After	Mollusca	96	Animalia	0.4146	0.031

Heatwave	Assay	OTU	Taxa	Indicator value	*p* value
After	Copepod 3	107	Calanoida	0.4146	0.032
After	Mollusca	128	Eukaryota	0.4146	0.034
After	Cnidaria	155	Eukaryota	0.4146	0.038
After	Copepod 3	44	Animalia	0.4125	0.016
After	Copepod 3	148	Decapoda	0.3902	0.033
After	Copepod 3	92	Gastropoda	0.3902	0.039
After	Copepod 3	138	Calanoida	0.3902	0.050

(v) Matched to vouchered specimen sequence

Table S3.14: Indicator species analysis for 17-month heatwave variation (Indval—Roberts, 2016)

Heatwave	Assay	OTU	Taxa	Indicator value	*p* value
Before	Cnidaria	165	Pythiales	0.7778	0.001
Before	Cnidaria	21	Hydrozoa	0.5234	0.001
Before	Copepod 1	74	*Paracalanus Indicus*	0.4892	0.002
Before	Mollusca	36	*Creseis sp.*	0.4705	0.004
Before	Universal	23	Syndiniales	0.4701	0.004
Before	Cnidaria	22	*Penilia avirostris*	0.4549	0.015
Before	Copepod 1	57	Arthropoda	0.4497	0.001
Before	Cnidaria	54	*Acartia negligens*	0.4460	0.016
Before	Cnidaria	87	Chlorophyta	0.4395	0.014
Before	Cnidaria	175	Animalia	0.4291	0.003
Before	Cnidaria	24	*Aglaura hemistoma*	0.4223	0.012
Before	Copepod 3	27	Clausocalanidae	0.4105	0.041
Before	Mollusca	137	Calanoida	0.4029	0.012
Before	Copepod 2	14	Calanoida	0.3980	0.015
Before	Mollusca	216	Arthropoda	0.3951	0.003
Before	Copepod 2	11	Arthropoda	0.3951	0.003
Before	Cnidaria	58	Arthropoda	0.3836	0.017
Before	Cnidaria	41	*Ophiura kinbergi*	0.3830	0.036
Before	Cnidaria	76	Chlorophyta	0.3652	0.012
Before	Mollusca	333	*Oncaea venusta typica*	0.3568	0.009
Before	Copepod 1	114	Arthropoda	0.3363	0.007
Before	Copepod 2	86	*Ophiura kinbergi*	0.3214	0.031
Before	Copepod 2	113	*Temora discaudata* (v)	0.2750	0.043
Before	Cnidaria	168	Plantae	0.2745	0.013
Before	Copepod 1	5	Mollusca	0.2617	0.030
Before	Mollusca	188	Pyramimonadophyceae	0.2333	0.029
Before	Copepod 1	110	Gastropoda	0.2222	0.025
Before	Mollusca	252	Polychaeta	0.2222	0.028
Before	Copepod 3	285	Calanoida	0.2222	0.029
Before	Copepod 1	133	Gastropoda	0.2222	0.036
Before	Cnidaria	127	Chlorophyta	0.1915	0.040
Before	Cnidaria	93	Leptothecata	0.1915	0.042
During	Copepod 2	35	Paracalanidae	0.4342	0.016
During	Copepod 3	35	Calanoida	0.4264	0.008
During	Copepod 3	130	*Subeucalanus sp.* (Two – 100%(v))	0.4043	0.019
During	Copepod 3	72	*Candacia truncate (v)*	0.3988	0.006
During	Copepod 3	52	*Eucalanus pseudattenuatus* (v)	0.3929	0.022
During	Copepod 3	83	*Acrocalanus gracilis*	0.3840	0.041
During	Mollusca	280	*Undinula vulgaris*	0.3829	0.010
During	Copepod 3	165	*Clausocalanus jobei (v)*	0.3713	0.040
During	Crustacea	31	Animalia	0.3663	0.004
During	Mollusca	17	*Lucifer sp.* (v)	0.3663	0.008
During	Copepod 2	105	*Acrocalanus gracilis* (v)	0.3634	0.010
During	Universal	48	*Subeucalanus pileatus*	0.3496	0.041
During	Copepod 2	107	Mollusca	0.3381	0.029
During	Copepod 1	103	Clausocalanidae	0.3363	0.022
During	Mollusca	115	*Creseis sp.*	0.3352	0.018

Heatwave	Assay	OTU	Taxa	Indicator value	*p* value
During	Universal	71	*Temora sp.*	0.3352	0.027
During	Mollusca	148	Prasinophyceae	0.3349	0.038
During	Copepod 3	121	Sagittidae	0.3245	0.050
During	Copepod 3	111	*Lucifer intermedius*	0.3150	0.010
During	Mollusca	215	Arthropoda	0.3043	0.042
During	Copepod 3	95	Arthropoda	0.3029	0.048
During	Copepod 1	122	*Undinula vulgaris*	0.2844	0.029
During	Crustacea	13	Animalia	0.2778	0.009
During	Universal	87	Chromista	0.2461	0.016
During	Mollusca	65	Decapoda	0.2461	0.026
During	Universal	61	Syndiniales	0.2461	0.028
During	Copepod 3	301	Potamididae	0.2314	0.022
During	Mollusca	179	Hexanauplia	0.2314	0.037
During	Cnidaria	105	Hydrozoa	0.2222	0.020
During	Copepod 2	64	*Candacia catula*	0.2222	0.022
During	Copepod 3	119	Chaetognatha	0.1915	0.046
After	Cnidaria	5	Prasinophyceae	0.7940	0.001
After	Mollusca	4	Eukaryota	0.6350	0.001
After	Cnidaria	16	Chlorophyta	0.6036	0.001
After	Mollusca	64	*Oncaea sp.* (Two – 100%)	0.5843	0.001
After	Copepod 1	4	Oncaea waldemari (v)	0.5455	0.001
After	Universal	13	Chlorophyta	0.4936	0.001
After	Copepod 1	19	Arthropoda	0.4802	0.003
After	Copepod 3	28	Muricidae	0.4603	0.006
After	Copepod 2	29	*Calocalanus styliremis* (v)	0.4474	0.015
After	Cnidaria	11	Hydrozoa	0.4346	0.001
After	Copepod 1	36	Arthropoda	0.4309	0.008
After	Cnidaria	36	Arthropoda	0.4227	0.021
After	Mollusca	27	Calanoida	0.4212	0.038
After	Copepod 1	9	Triconia sp.	0.4208	0.025
After	Copepod 1	17	Arthropoda	0.4133	0.020
After	Cnidaria	104	Chlorophyta	0.4100	0.006
After	Copepod 1	20	Arthropoda	0.4094	0.015
After	Copepod 3	148	Decapoda	0.4091	0.010
After	Copepod 3	44	Animalia	0.4047	0.012
After	Mollusca	30	*Cacozeliana granarium*	0.3971	0.044
After	Copepod 1	7	Arthropoda	0.3945	0.026
After	Copepod 3	86	*Calocalanus styliremis*	0.3821	0.042
After	Copepod 1	84	Arthropoda	0.3746	0.014
After	Copepod 1	54	Arthropoda	0.3701	0.044
After	Copepod 1	42	Arthropoda	0.3609	0.013
After	Copepod 3	190	*Calocalanus sp.*	0.3403	0.018
After	Copepod 1	27	Acartiidae	0.3400	0.040
After	Copepod 1	31	Ataxocerithium	0.3296	0.050
After	Cnidaria	155	Eukaryota	0.3140	0.030
After	Cnidaria	25	Gastropoda	0.3086	0.044
After	Copepod 1	47	Arthropoda	0.2857	0.024
After	Copepod 3	315	Calanoida	0.2724	0.035
After	Mollusca	309	Mollusca	0.2500	0.028

Table S3.15: Alternative linear models for Assemblage and Richness

Assay used	OTU diversity test	Most parsimonious model (R^2)	Alternative Model 1	Alternative Model 2	Alternative Model 3	Alternative Model 4	Alternative Model 5
Cnidaria	Assemblage	SST, Salinity, Silicate (0.162)	Silicate	SST, Silicate	SST, Salinity, Silicate, Phosphate	SST, Salinity, Silicate, Nitrate, Phosphate	SST, Salinity, Silicate, Nitrate, Phosphate, Ammonium
	AIC	404.45	405.92	405.12	405.02	405.51	406.10
	Richness	SST, Silicate (0.112)	Silicate	SST, Silicate, Phosphate	SST, Silicate, Phosphate, Ammonium		
	AIC	447.24	447.30	447.41	449.05		
Copepod 1	Assemblage	SST, Salinity, Silicate (0.155)	Silicate	SST, Salinity, Silicate, Nitrate, Phosphate, Ammonium	SST, Salinity, Silicate, Nitrate, Phosphate, Ammonium	SST, Salinity, Silicate, Nitrate, Phosphate, Ammonium	
	AIC	404.62	405.3	405.02	405.33	406.17	
	Richness	Salinity (0.067)	Salinity, Phosphate				
	AIC	451.13	452.61				
Copepod 2	Assemblage	SST, Salinity, Silicate (0.230)	Salinity, Silicate	SST, Salinity, Silicate, Ammonium			
	AIC	382.78	383.94	383.59			
	Richness	Salinity, Silicate (0.309)	Salinity, Silicate, Ammonium	Salinity, Silicate, Phosphate, Ammonium			
	AIC	427.16	427.60	429.03			
Copepod 3	Assemblage	SST, Salinity, Silicate (0.227)	Salinity	SST, Salinity	SST, Salinity, Silicate, Ammonium	SST, Salinity, Silicate, Phosphate, Ammonium	
	AIC	383.36	385.25	383.83	383.79	384.49	
	Richness	SST, Salinity, Ammonium (0.392)	SST, Salinity	SST, Salinity, Phosphate, Ammonium			
	AIC	492.79	492.90	494.31			
Crustacea	Assemblage	SST, Salinity (0.098)	Silicate	SST, Salinity, Ammonium	SST, Salinity, Silicate, Ammonium	SST, Salinity, Silicate, Phosphate, Ammonium	

Assay used	OTU diversity test	Most parsimonious model (R^2)	Alternative Model 1	Alternative Model 2	Alternative Model 3	Alternative Model 4	Alternative Model 5
	AIC	405.52	405.97	405.89	406.61	407.39	
	Richness	SST, Ammonium (0.183)	SST, Salinity, Ammonium	SST, Salinity, Silicate, Ammonium			
	AIC	376.85	377.39	377.43			
Fish	Assemblage	SST, Salinity, Ammonium (0.147)	Salinity	SST, Salinity	SST, Salinity, Phosphate, Ammonium	SST, Salinity, Silicate, Nitrate, Phosphate	SST, Salinity, Silicate, Nitrate, Phosphate, Ammonium
	AIC	427.43	428.33	427.77	428.07	428.25	428.9
	Richness	Nitrate, Salinity, Silicate, Phosphate (0.251)	Nitrate, Silicate	Nitrate, Salinity, Silicate	SST, Nitrate, Salinity, Silicate, Phosphate		
	AIC	262.88	263.95	263.88	264.82		
Mollusca	Assemblage	SST, Salinity, Silicate (0.197)	SST, Salinity	SST, Salinity, Silicate, Ammonium	SST, Salinity, Silicate, Phosphate, Ammonium	SST, Salinity, Silicate, Nitrate, Phosphate, Ammonium	
	AIC	388.46	389.22	388.85	389.25	390.17	
	Richness	SST (0.061)	SST, Ammonium	SST, Silicate, Ammonium			
	AIC	499.91	500.94	501.65			
Universal	Assemblage	SST, Salinity, Silicate (0.140)	Salinity	Salinity, Silicate	SST, Salinity, Silicate, Phosphate	SST, Salinity, Silicate, Phosphate, Ammonium	SST, Salinity, Silicate, Nitrate, Phosphate, Ammonium
	AIC	371.36	372.12	371.4	370.95	370.95	371.89
	Richness	SST, Salinity, Phosphate (0.212)	SST, Nitrate, Salinity, Phosphate				
	AIC	374.71	376.55				

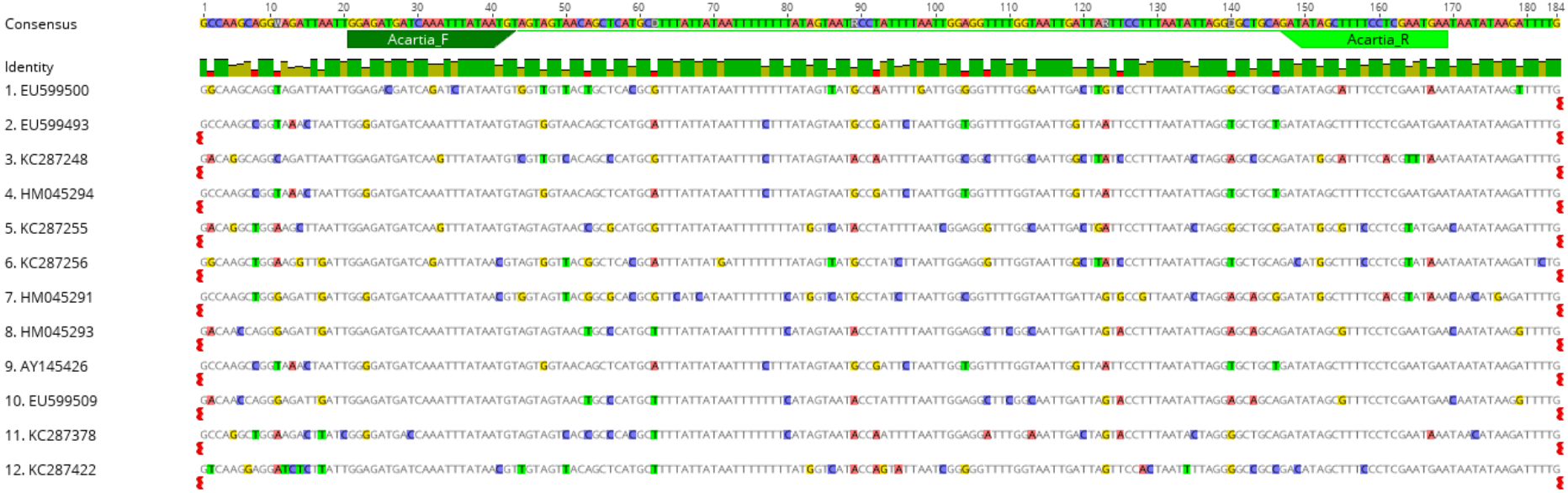


Figure S3.4: Example of a consensus alignment used to create the assays used in this study. This COI alignment resulted in the Copepod 3 assay (Kearse et al. 2012).

Table S3.16 Metabarcoding PCR assays developed and tested for this study.

PCR assay	Primer set used	Target Taxa	Gene	Primer sequence	Amplicon length (bp)	Reference	Assay T_a (°C)	Mostly detected
Copepod 3	Acartia F Acartia R	Calanoida	COI	5′ GGRGAYGATCARRTYTATAAYGT 3′ 5′ TTYATWCGWGGAAAHGCYATRTC 3′	~103	This study	52	Malacostraca, Hexanauplia, Actinopterygii
Acrocalanus	Acrocalanus F Acrocalanus R	Calanoida	COI	5′ CTGTTTAYCCWCCTCTTTCTAG 3′ 5′ TCTARTAAYATDCCAAATACTCGT 3′	~111	Pilot study	50	Hexanauplia
Branchiostoma	Branchiostoma F Branchiostoma R	Leptocardii	COI	5′ CRGAYATRGCKTTYCCYCG 3′ 5′ AAAATTGAYGAYACMCCWGC 3′	~169	Pilot study	50	Mollusca
Calanus	Calanus F Calanus R	Calanoida	COI	5′ GCWTCYGTNGAYTTYGCWAT 3′ 5′ CCSCCCACATCAYAMAATG 3′	~212	Pilot study	51	Very little
Calocalanus	Calocalanus F Calocalanus R	Calanoida	COI	5′ CTGGDTCNTTRATTGGRGATG 3′ 5′ GAHAAAGGDGGRTAAACWGTTC 3′	~225	Pilot study	51	Hexanauplia
Candacia	Candacia F Candacia R	Calanoida	COI	5′ GGVRSATSAGTDGACTTTGC 3′ 5′ GCAAANAGAGGYATWCGNTC 3′	~97	Pilot study	51	Hexanauplia
Cnidaria	Cnidaria F Cnidaria R	Cnidarians	COI	5′ CATGATHTTYTTCWTDGTMATGCC 3′ 5′ GTYCAWCCWGTWCCWRCYCC 3′	~145	This study	52	Cnidaria, Echinodermata
Copilia	Copilia F Copilia R	Cyclopoida	COI	5′ CGCAATCTTCTCTCTACACC 3′ 5′ GGTAATGAGAGGAGAAGTAGG 3′	~124	Pilot study	53	*Copilia mirabilis*
Creseis	Creseis F Creseis R	Gastropoda	COI	5′ CATTCTGACTHCTHCCTCCWTC 3′ 5′ GCMCCHAGAATAGAAGAAATACC 3′	~136	Pilot study	53	Mollusca
Crust 16S	Crust16S_F(short) Crust16S_R(short)	Crustaceans	16S rRNA	5′ GGGACGATAAGACCCTATA 3′ 5′ ATTACGCTGTTATCCCTAAAG 3′	~170	Pilot study	51	Malacostraca
Cyttarocylis	Cyttarocylis F Cyttarocylis R	Ciliophora	18S rRNA	5′ CGCGTAAATTACCCAATCCTG 3′ 5′ CTAACAGAAATCCAACTACGAGC 3′	~169	Pilot study	56	Chromis
Doliolum	Doliolum F Doliolum R	Thaliacea	18S rRNA	5′ GGGATGCGTGCTTTTATC 3′ 5′ CTTGGATGTGGTAGCCG 3′	~196	Pilot study	52	Very little
Eucalanus	Eucalanus F Eucalanus R	Calanoida	COI	5′ GTAGARAGAGGNGCHGGWAC 3′ 5′ GCAGTAATVACKACDGCYCA 3′	~184	Pilot study	52	Hexanauplia

PCR assay	Primer set used	Target Taxa	Gene	Primer sequence	Amplicon length (bp)	Reference	Assay T_a (°C)	Mostly detected
Fish 16S	Fish16sF/D 16s2R (degenerate)	Fish	16S rRNA	5′ GACCCTATGGAGCTTTAGAC 3′ 5′ CGCTGTTATCCCTADRGTAACT 3′	~200	F- Berry et al. (2017) R-Deagle et al. (2007)	54	Actinopterygii
Mollusca	Limacina F Limacina R	Mollusca	COI	5′ TAATTGGNGGVTTTGGRAAYTG 3′ 5′ GTTCAHCCTRAYCCTRCNCC 3′	~118	This study	52	Hexanauplia, Cnidaria, Mollusca
Copepod 2	Lucicutia F Lucicutia R	Calanoida	COI	5′ CCHGAYATAGCTTTYCCHCG 3′ 5′ GAAAAAATTGCAAAATCTACDGATC 3′	~134	This study	52	Hexanauplia, Mollusca
Lucifer	Lucifer F Lucifer R	Decapoda	COI	5′ GGAGAYGAYCARATTTATAATGTAG 3′ 5′ GAGGRAAWGCYATATCAGGAG 3′	~96	Pilot study	51	Hexanauplia
Oithona	Oithona F Oithona R	Cyclopoida	COI	5′ CCAGATATAGCHTTYCCHCG 3′ 5′ GAAGAHACHCCYGCYAARTG 3′	~163	Pilot study	52	Hexanauplia
Oncaea	Oncaea F Oncaea R	Cyclopoida	COI	5′ TTGGSKGKTTYGGWAATTG 3′ 5′ GCTAARTGAAGWSHAAAAATRG 3′	~194	Pilot study	48	Hexanauplia
Paracalanus	Paracalanus F Paracalanus R	Calanoida	COI	5′ CCRTTAATAYTAGGAGCAGC 3′ 5′ CTAAAATWGAACTWACWCCTGC 3′	~187	Pilot study	51	Hexanauplia
Pontellidae	Pontellidae F Pontellidae R	Calanoida	COI	5′ GRGCAGGWACDGGDTGRAC 3′ 5′ CGATCYAAAATTATYCCAAAHACWCG 3′	~133	Pilot study	54	Hexanauplia
S_Ceph 16S	S_Cephalopoda_F S_Cephalopoda_R	Cephalopoda	16S rRNA	5′ GCTRGAATGAATGGTTTGAC 3′ 5′ TCAWTAGGGTCTTCTCGTCC 3′	~70	Peters et al. (2014)	50	Mollusca Crustacea
Copepod 1	Triconia F Triconia R	Cyclopoida	COI	5′ CAGGVTCTTTAYTWGGRGATG 3′ 5′ AAAATCTTATATTATTTARBCGRGG 3′	~131	This study	49	Hexanauplia, Mollusca
Urchin	Urchin F Urchin R	Echinodermata	16S rRNA	5′ GACGAGAAGACCCTGDCG 3′ 5′ CGCTGTTATCCCTRCGGTAAC 3′	~200-230	Pilot study	55	Echinodermata
WSP	WSP F WSP R	Decapoda	COI	5′ AGWYTWATTATYCGRGCWGA 3′ 5′ TCARTTWCCAAATCCWCC 3′	~107	Pilot study	48	Malacostraca

"F" refers to the forward primer; "R" refers to the reverse primer.

ALA. 2016. *Atlas of Living Australia website* [Online]. http://www.ala.org.au. [Accessed September 2016].

ANDERSON, M., GORLEY, R. N. & CLARKE, R. K. 2008. *Permanova+ for Primer: Guide to Software and Statisticl Methods*, Primer-E Limited.

BERRY, T. E., OSTERRIEDER, S. K., MURRAY, D. C., COGHLAN, M. L., RICHARDSON, A. J., GREALY, A. K., STAT, M., BEJDER, L. & BUNCE, M. 2017. DNA metabarcoding for diet analysis and biodiversity: A case study using the endangered Australian sea lion (*Neophoca cinerea*). *Ecol Evol,* 7**,** 5435-5453.

BUREAU OF METEOROLOGY, 2016. *Australian climate variability & change - Time series graphs* [Online]. http://www.bom.gov.au/tmp/cc/sst.sw.0112.23911.png: Australian Government. [Accessed 20 December 2017].

DAVIES, C. H., ARMSTRONG, A. J., BAIRD, M., COMAN, F., EDGAR, S., GAUGHAN, D., GREENWOOD, J., GUSMÃO, F., HENSCHKE, N., KOSLOW, J. A., LETERME, S. C., MCKINNON, A. D., MILLER, M., PAUSINA, S., PALOMINO, J. U., ROENNFELDT, R.-L., ROTHLISBERG, P., SLOTWINSKI, A., STRZELECKI, J., SUTHERS, I. M., SWADLING, K. M., TALBOT, S., TONKS, M., TRANTER, D. H., YOUNG, J. W. & RICHARDSON ANTHONY, J. 2014. Over 75 years of zooplankton data from Australia. *Ecology,* 95**,** 3229-3229.

DEAGLE, B. E., GALES, N. J., EVANS, K., JARMAN, S. N., ROBINSON, S., TREBILCO, R. & HINDELL, M. A. 2007. Studying Seabird Diet through Genetic Analysis of Faeces: A Case Study on Macaroni Penguins (*Eudyptes chrysolophus*). *PLoS ONE,* 2**,** e831.

KEARSE M, MOIR R, WILSON A, STONES-HAVAS S, CHEUNG M, STURROCK S, ET AL. 2012. Geneious Basic: An integrated and extendable

desktop software platform for the organization and analysis of sequence data. *Bioinformatics*. 28(12):1647-9.

PETERS KJ, OPHELKELLER K, BOTT NJ, DEAGLE BE, JARMAN SN, GOLDSWORTHY SD. 2014. Fine-scale diet of the Australian sea lion (*Neophoca cinerea*) using DNA-based analysis of faeces. *Marine Ecology*. 1-21.

POCHON, X., BOTT, N. J., SMITH, K. F. & WOOD, S. A. 2013. Evaluating Detection Limits of Next-Generation Sequencing for the Surveillance and Monitoring of International Marine Pests. *PLOS ONE,* 8**,** e73935.

ROBERTS, D. W. 2016. labdsv: Ordination and Multivariate Analysis for Ecology. R package version 1.8-0.

THOMPSON, H. 1948. Pelagic tunicates of Australia.

3.11 Close

THIS was the first single-site, long-term, multi-assay, eDNA metabarcoding study involving plankton. The results provide a rare glimpse into the Rottnest Island zooplankton community and how it responds to different climatic stressors. The utility and potential of both the methodologies and the assays as a 'tool kit' for marine environments was confirmed.

While some assays detected taxa that responded significantly to seasonal and/or annual changes, other assays revealed how the planktonic community reacted to the stress of the 2011 and 2012 heatwaves. Hence the value of the multi-assay approach was established —had the study been limited to only one or two assays either of these responses might have been missed. The results also emphasise the significance of the long-term ongoing collection and use of time-stamped data. If the analysis been restricted to a few sampling points, a good snapshot of zooplankton diversity might have been obtained. Yet an undersized study would lack the statistical power to identify the key temporal changes within the community.

The results suggested that heat-induced stress in the form of seasonal and heatwave anomalies had profound and—in the case of the heatwaves—potentially lasting effects on the structure of the zooplankton population. The inference being that sea surface temperature (SST) was a strong contributor to these changes.

The statistical models for each assay substantiated the importance of the abiotic factors to the overall differences in both the zooplankton OTU composition—Assemblage—and total genetic diversity—Richness—between the time periods. Abiotic factors accounted for between 7 – 39% of the variation in Richness and 10 – 23% of Assemblage variation, depending on the target taxa. SST and salinity were found to be most significant factors across the majority of assays.

Given the imminent threat of global warming for the future of marine biomes, the findings from this research are disturbing. They warn that, in response to warming, significant changes are already taking place—at least within localised areas.

These findings emphasise the need for continued temporal and spatial eDNA biobanks to be established (Jarman et al., 2018). Such a program would allow for the long-term monitoring of base lines and changes and, in consequence, provide the opportunity for answers to future questions.

As detailed as these findings were, this study is restricted to data from the Rottnest Island NRS. IMOS provided additional samples—a total of 90 seasonal plankton samples—taken from all nine NRS stations over a period of three years. Chapter four uses these samples and the 'tool kit' from Chapter three to endeavour to validate the methodologies further—investigating both spatial and temporal variations.

BERRY, T. E., 2013. Development of molecular tools for dietary analysis: A tail end look at Western Australia's aquatic ecosystems. *Murdoch University; School of Veterinary and Life Sciences.*

CAPUTI, N., JACKSON, G. AND PEARCE, A. 2014. The marine heat wave off Western Australia during the summer of 2010/11 - 2 years on. *Fisheries Research Report No 250.*

JARMAN, S. N., BERRY, O. AND BUNCE, M. et al. 2018. The value of environmental DNA biobanking for long-term biomonitoring. *Nature Ecology & Evolution* 2(8): 1192-1193.

KEARSE, M., MOIR, R., WILSON, A., STONES-HAVAS, S., CHEUNG, M., STURROCK, S., BUXTON, S., COOPER, A., MARKOWITZ, S., DURAN, C., THIERER, T., ASHTON, B., MEINTJES, P. & DRUMMOND, A. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics,* 28**,** 1647-1649.

LYNCH, T. P., MORELLO, E. B., EVANS, K., RICHARDSON, A. J., ROCHESTER, W., STEINBERG, C. R., ROUGHAN, M., THOMPSON, P., MIDDLETON, J. F., FENG, M., SHERRINGTON, R., BRANDO, V., TILBROOK, B., RIDGWAY, K., ALLEN, S., DOHERTY, P., HILL, K. & MOLTMANN, T. C. 2014. IMOS National Reference Stations: a continental-wide physical, chemical and biological coastal observing system. *PLoS One,* 9**,** e113652.

PEARCE, A. F. & FENG, M. 2013. The rise and fall of the "marine heat wave" off Western Australia during the summer of 2010/2011. *Journal of Marine Systems,* 111-112**,** 139-156.

PETERS, K. J., OPHELKELLER, K., BOTT, N. J., DEAGLE, B. E., JARMAN, S. N. & GOLDSWORTHY, S. D. 2014. Fine-scale diet of the Australian sea lion (*Neophoca cinerea*) using DNA-based analysis of faeces. *Marine Ecology***,** 1-21.

CHAPTER – FOUR

A three-year, Australia-wide, environmental DNA study reveals spatial and temporal patterns in marine biodiversity

A Zooplankton makes
meals of plants, then it becomes
a dinner for one.

'The essential link' – Tina E Berry (2017)

4.1 Prelude

CHAPTER Three illustrates the importance of extended longitudinal environmental DNA (eDNA) studies to the ongoing monitoring of marine biodiversity. The use of multi-gene metabarcoding, using high throughput sequencing, not only distinguished seasonal changes within the zooplankton community, but also tracked biotic responses to the heatwave anomaly experienced at the site from 2011-2012. Nevertheless, the research in Chapter Three was limited to one site, the Rottnest Island National Research Station (NRS).

This chapter details the expansion of the study to include all nine Integrated Marine Observing System (IMOS) NRS situated around Australia. The placement of these sites (Figure 4.1) ranges from the tropical waters of Darwin, across the Tropic of Capricorn to the temperate waters of Maria Island. Their geographical isolation presents a unique opportunity to assess how the zooplankton communities vary spatially. The broad range of sites also allows for investigation of the influence that site based abiotic changes have on planktonic communities. Given the cost and logistics of using six metabarcoding assays over nine sites, the sampling interval was limited to seasonal sampling across a three-year window, from 2012-2014 inclusive.

IMOS provided access to 90 seasonal plankton community samples, preserved at -80° C specifically for genetic research. While the samples were taken across a period of three years, two of the NRS had ceased sampling plankton for molecular purposes in 2013 and some stations did not sample for several of the relevant seasons. The first aim of this research was to determine whether, using six - twelve samples per site and six assays, significant spatial differences in the zooplankton communities could be detected. A further goal was to determine whether this limited seasonal sampling would be enough to detect the temporal changes within each site. The study design generated over 500 metabarcoding datasets; a total of in excess of 25 million sequences and over 500 assignments to taxa.

An alternative data pipeline was devised, and utilised, in an effort to streamline analysis time. This new methodology still produced an immense amount of data including some surprising taxonomic results that are interpreted in a spatial context.

Chapter Four is a manuscript in the final stages of preparation for submission to *Molecular Ecology* with minimal changes made to the format to fit the style of this thesis. It is expected that this paper will be submitted for publication prior to the end of 2020.

4.1.1 Acknowledgements

I thank the Integrated Marine Observing System (IMOS) for providing access to the samples and abiotic data used during this study. Supported by the Australian Government, IMOS is a national collaborative research infrastructure, which is operated by a consortium of institutions as an unincorporated joint venture, with the University of Tasmania as Lead Agent. An Australian Government Training Program Scholarship and the resources provided by the Pawsey Supercomputing Centre, which is funded by the Governments of Australia and Western Australia, also supported this work. M.B. acknowledges the support of ARC Linkage projects (LP160100839 and LP160101508) to explore marine metabarcoding applications.

4.1.2 Author contributions

T.E.B., O. B., A.J.R. and M.B. conceived and designed the study. C.D. and A.J.R. facilitated access to the samples and abiotic data. T.E.B., M.L.C and M.P. refined the approach and produced the data. T.E.B., B.S. and A.J.R designed and produced the statistical analysis. T.E.B., S.J., M.B., M.L.C and B.S. discussed the results in preparation of the manuscript. All authors will be involved in the final editing of the manuscript.

4.1.3 Author Declarations

The Authors declare they have no competing interests.

A three-year, Australian-wide, environmental DNA study reveals spatial and temporal patterns in marine biodiversity

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Prepared for submission to *Molecular Ecology*

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4.2 Abstract

THE exploration of total biodiversity is vital to the holistic understanding of functioning ecosystems. Environmental DNA (eDNA) is increasingly employed to survey biodiversity, and is of greatest use where conventional methods fail to reveal sufficient taxonomic depth within the biome of interest. While recent eDNA studies have compared spatial differences within terrestrial biodiversity, marine environments—particularly with respect to zooplankton—have received far less investigative attention. Metabarcoding of eDNA is a relatively recent innovation, consequently extensive temporal and spatial marine sample sets suitable for eDNA extraction are rare. Here we use three years of seasonal bulk-plankton samples, taken from nine Australia-wide communities (n=90), to produce over 25 million sequences and show that significant spatial differences can be mapped using eDNA metabarcoding methodologies. A cross validation test, of the spatial fidelity of the data, resulted in an average—sample to site—reassignment score of 80%. The abiotic data collected also enabled us to track how these factors correlate with community composition. Our plankton samples also revealed new insights into the marine biomes—from invasive species to the detection of Humpback whales (*Megaptera novaeangliae*). These cetacean sequences originated from a total of six samples and four sites, and were obtained during the whales' usual migration patterns. This work builds on a previous investigation of temporal variations in a single zooplankton population using eDNA metabarcodes, and provides a comprehensive study of spatial variation in marine metazoan community composition. Importantly, the results provide a guide to better understand what eDNA might offer ongoing marine biomonitoring programs that employ temporal and spatial sampling.

4.3 Introduction

EVIDENCE from across tens of thousands of years confirms that terrestrial animal extinctions and extirpations have accelerated with human contact. However, until recently, the protection offered by the marine environment minimised the exploitation of its fauna—particularly in deep seas and far ocean regions (see McCauley et al., 2015). This protection ended with the introduction of industrialisation and advent of commercial fisheries (Lotze et al., 2006, McCauley et al., 2015). Anthropogenic climate change is an additional contemporary threat, and transformations of marine environments in response to rising atmospheric and ocean temperatures are expected to have profound ecological (Edwards and Richardson, 2004, Gattuso et al., 2015, Griffiths et al., 2017), and financial and societal consequences (Beaugrand et al., 2002, Wernberg et al., 2016). Ocean acidification, caused by increased atmospheric CO_2 concentrations, compounds these effects (Richardson, 2009, Gattuso et al., 2015). Together these burdens place an impetus for the effective management and protection of our marine life for future generations (Edwards et al., 2010, Gattuso et al., 2015).

Most monitoring programs concentrate on larger bodied marine organisms, such as fish (Richardson, 2009, Lenanton et al., 2017) and marine mammals. Yet these animals, for the most part, comprise the higher end of the marine food chain and respond relatively slowly to external pressures on ecosystems. In contrast, zooplankton are crucial to the wellbeing of the Earth's oceans and the species composition of zooplankton responds rapidly to changing conditions (Richardson, 2009). They may be herbivorous, omnivorous or carnivorous, but their roles as the critical trophic link between the marine autotrophs and higher order animals makes monitoring their species composition valuable (Richardson, 2009). Accordingly, for almost 90 years the mass and composition of planktonic communities in the Northern Hemisphere have been used as 'barometers' of marine ecosystem health (Edwards et al. 2010).

Long-term biomonitoring of ocean communities is rare, but of great value in determining baselines of ecosystem composition (Richardson et al., 2012, Edwards et

al., 2010). Historically, the complexity of zooplankton communities (Richardson, 2009) and the size of their individual members have presented an ongoing challenge for researchers and thus ongoing spatial and temporal monitoring is restricted (Deagle et al., 2017). Morphology has been the chief method for identification of zooplankton to date; but the process is time consuming, and the proportion of collected animals that are in a condition for identification is limited (Deagle et al., 2017, Lindeque et al., 2013).

The collection of community plankton samples analysed holistically is classified as an eDNA substrate (Taberlet et al. 2018). Contemporary research using (eDNA) metabarcoding technology has demonstrated the ability to extract useful genetic data from numerous marine environmental substrates (for example; Deagle et al., 2005, Casper et al., 2007, Guardiola et al., 2015, Andruszkiewicz et al., 2017, Morard et al., 2017, Berry et al., 2017, Stat et al., 2017, Hardy et al., 2017—see thesis Appendix). However only a small number of metabarcoding biodiversity studies, using plankton as an eDNA substrate, have been undertaken (Lindeque et al., 2013, Gimmler et al., 2016, Harvey et al., 2017, Deagle et al., 2017, Alberti et al., 2017). This is in part because many existing barcodes lack the balance between the taxonomic coverage and resolution required to meet the challenge of this highly diverse substrate (Clarke et al., 2017, Brown et al., 2015, Lindeque et al., 2013).

Recently we demonstrated that a multi-gene approach to metabarcoding could be used to map temporal changes in a zooplankton community in response to seasonal and heatwave conditions across five years (Berry et al. 2019—Chapter Three). Here we take six metabarcoding assays (Table S4.1) and apply them to 90 seasonally collected plankton samples taken from nine Integrated Marine Observing System (IMOS) stations from around Australia over a three year period (Figure 4.1). In doing so we look to test the capacity of eDNA to reveal not only the spatial and seasonal differences in the zooplankton communities, but also to explain some of these differences by investigating links with corresponding abiotic data.

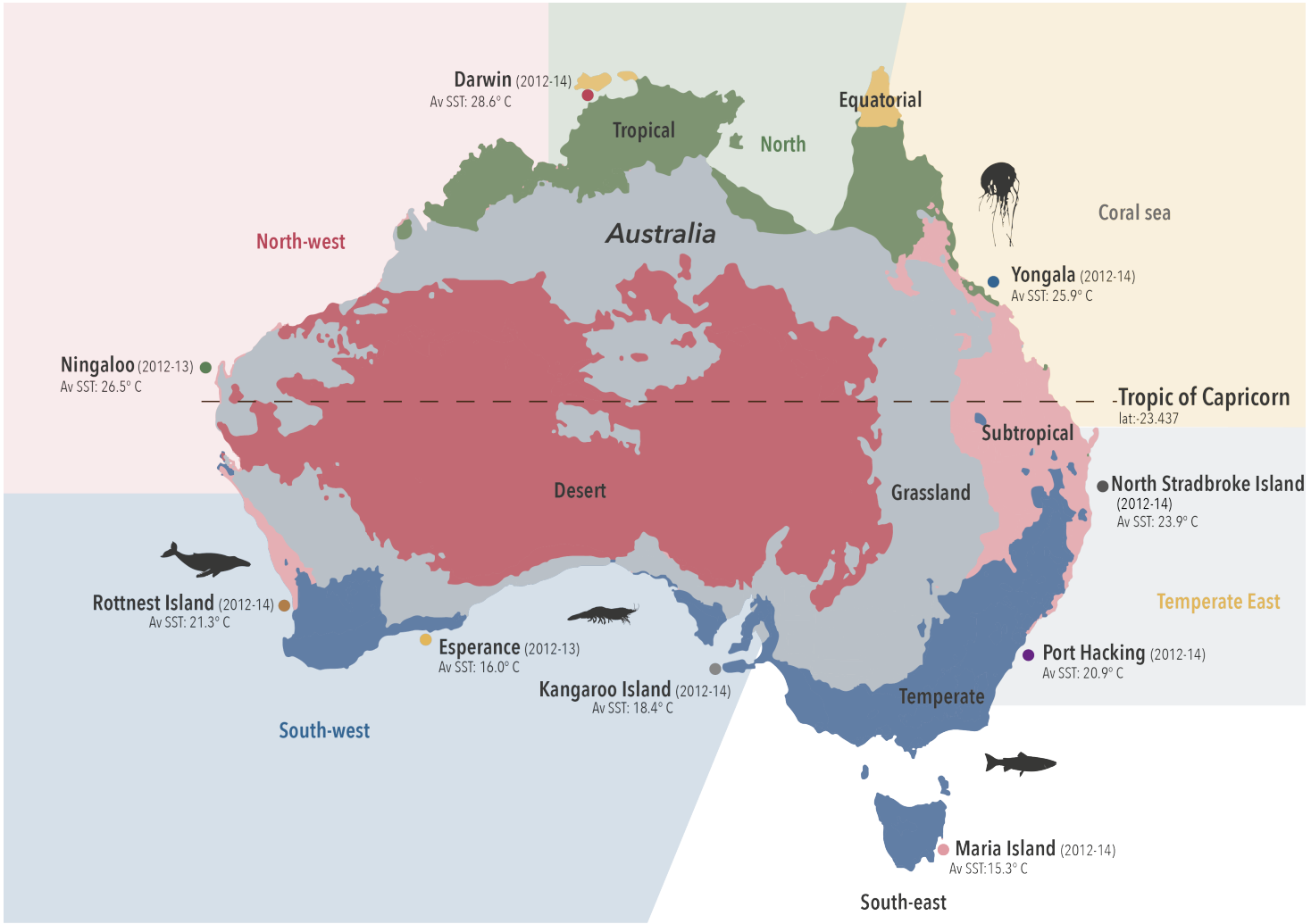

Figure 4.1: The Australian Integrated Marine Observing System (IMOS) sampling sites. The map includes; adapted Köppen climate classifications, marine biogeographical areas, and for each site, the sampling years (in brackets) and average sea surface temperatures (Av SST).

4.4 Materials and methods

4.4.1 Sampling details & subsequent DNA extraction

Samples were collected from nine IMOS NRS (Figure 4.1) across 2012 - 2014. Three sites are situated north of the Tropic of Capricorn and six are south. Each state and territory has at least one station—with the exception of Victoria and the Australian Capital Territory) —Western Australia hosts three and Queensland has two. Sampling has taken place at most of these sites since 2008 (Lynch et al., 2014).

Plankton samples were collected via a vertical plankton tow using a 0.6 m wide, 3 m long drop net (Heron, 1982) with a100-micron mesh, which falls at 1 ms-1. The seabed depth at each site varies (Lynch et al., 2014) —the sample depth is 5 m above the seabed. Plankton is collected on the downward tow only—the net is closed as it is recovered to the surface.

Seawater was used to wash the samples into the codend of the drop net for transfer to the sample jar. After collection the samples were iced until stored at -80°C immediately on return to the lab. After subsampling they were kept at -20°C.

Plankton samples were thawed and homogenised with a hand held blender (OMNI Tip™ Homogenizer) and a hard tissue probe. About 40 µL of each sample was then digested and extracted using DNAeasy Blood and Tissue kit (Qiagen) using the tissue protocol, with twofold quantities of the reagents used before the washing stage and a double elution of AE buffer (200 µL total). Negative controls were also produced during the extraction stages to be used throughout data production. DNA extracts were kept at -20°C. All samples were extracted and processed within laboratories especially designed for trace and environmental DNA.

4.4.2 Amplification of barcodes, eDNA library build & barcode sequencing

Six assays (Table S4.1) were applied to a one in ten dilution of the DNA extracts. For each of these assays, the DNA extracts were assigned fusion tagged primers incorporating assay specific primers, Illumina adaptor sequences and unique combinations of six to eight base MID (Multiplex IDentifier) tags (over 500 unique combinations). The MID tags identify the sequences to a particular assay and sample. To avoid cross contamination, the MID tag combinations had not previously been used for marine samples. Each PCR tagging reaction comprised: 1 x Taq Gold buffer (Applied Biosystems [ABI], USA), 2 nM MgCl2 (ABI, USA), 0.4 mg/mL BSA (Fisher Biotec, Australia), 0.25 mM dNTPs (Astral Scientific, Australia), 0.4 µM each of forward and reverse primers (Integrated DNA Technologies, Australia), 0.6 µL of 1/10,000 SYBR Green dye (Life Technologies, USA) and 1 U of Taq polymerase Gold (ABI, USA), 2 µL of DNA, and made up to 25 µL with PCR grade water. PCR master-mixes with tagged primers were prepared with-in an ultraclean environment to prevent contamination prior to the addition of DNA. PCR reactions were duplicated and cycled at; 95°C for 5 min followed by 50 cycles of 95°C for 30 s, the primer specific Ta (annealing temperature; Table S4.1) for 30 s and 72°C for 45 s, and a final extension of 72°C for 10 min. Tagged amplicons were combined in roughly equimolar concentrations to produce several sequencing libraries. The libraries were filtered for size using a Pippin Prep (Sage Sciences, MA, USA) instrument (2% Agarose cassette with ethidium bromide) and quantified with a Qubit fluorometer (ThermoFisher, Aus). All sequencing was completed on Illumina's MiSeq® (CA, USA) using the manufacturer's protocol (300 cycle V2 reagents and standard flow cell).

4.4.3 Production of Operational Taxonomic Units (OTUs)

The process of creating OTUs was repeated for each assay. Sequences were separated to their sample of origin by their MID tags using Geneious R8 (Kearse et al., 2012). Initial filtering was conducted by ensuring the MID tags, gene specific primers and sequencing adapters, were all present in each sorted sequence without error. Those sequences failing this were removed from future analyses. The primers, adaptors and MID tags were

detached from the remaining sequences. Each sequence was renamed to reflect its sample of origin. The filtered, trimmed and renamed sequences were then combined into one large assay specific file for further quality filtering and clustering into OTUs.

USEARCH v8 (Edgar, 2010) was used for quality filtering and the clustering of OTUs. Sequences were filtered using a fastq filter—E_max > 0.5—and formed into groups of identical sequences, removing those with an abundance of < 0.1% of the total number of unique sequences across all samples. OTUs were clustered using a 97% similarity threshold, a procedure that removes chimeric sequences. Low abundant filtered sequences were then mapped back on to existing OTUs to ensure the inclusion of all relevant data. Any low abundant unmapped amplicons were discarded from further analysis. This process, while potentially eliminating some scarce taxa, ensures the removal of possible erroneous amplicons. The OTUs were then assigned to samples and converted to presence/absence data, ready for potential identification and statistical analysis. The resulting OTU sequence data will be available for download on Data Dryad.

4.4.4 Generation of taxonomic assignment

OTU consensus sequences were searched against the National Center for Biotechnology Information (NCBI) GenBank nucleotide database (Nov 3 2017; Benson et al., 2014) using BLASTn (Basic Local Alignment Search Tool (Altschul et al., 1990)) with the default parameters and a reward of 1.

The output files from the search were imported into MEGAN v5 (METaGenome ANalyzer; Huson et al., 2011) and visualised with the LCA (lowest common ancestor) parameters: min bitscore 100.0. The reports were limited to best 5% matches. Taxonomic assignment was contemplated only where the whole of the OTU consensus sequence was matched. Taxa identified were combined into one table. However taxa identified more than once in a sample by OTUs from more than one assay were included in the table only once.

4.4.5 Statistical analysis of OTUs

Statistical analyses, were performed using PERMANOVA+ (Anderson et al., 2008) add on for Primer 7 (Clarke and Gorley, 2015) and R (R Core Team, 2015) with labdsv (Roberts, 2016), and vegan (Oksanen et al., 2016). With the exception of *indval* (labdsv), statistical analysis were performed on the presence/absence OTU data matrix for the sequences obtained for each assay, thus allowing for all available genetic information to be taken into consideration. A total of 90 samples were used for analysis.

At each data point, Richness—number of OTUs: univariate—and Assemblage—genetic composition: multivariate—were assessed for site, yearly and seasonal effects using PERMANOVA (Anderson, 2001). To illustrate site-based patterns, nonmetric multidimensional scaling (nMDS) plots were formed in R (vegan). Power analyses for season were conducted in R on data produced in PERMANOVA (Anderson et al., 2008). A further cross validation test (CAP: Anderson et al. 2008) between the sites was applied to the OTU assemblages. The indicator species that were characteristic of each site were identified using *indval* analyses in R (*indval*; labdsv)—this analysis was applied to the presence/absence of taxa identified.

The roles of abiotic variables were explored using linear models in the differences of both the multivariate OTU assemblage, and the univariate OTU richness recorded from each assay. Multivariate analyses were performed using distance based linear models (DistLM) in PERMANOVA+. Bray-Curtis similarity matrices were created from the presence/absence OTU data. The abiotic variables sea surface temperature (SST), salinity, silicate, nitrate, and phosphate were available for selection by the model. The 'best' selection technique and the AIC selection gauge were used to choose the model that best explained the variation in the OTU assemblage that was recorded for each assay. The best alternative models within 2 AIC of the selected model were also reported (Table S4.2).

For analysis of the univariate OTU richness documented using each assay, generalised linear models (GLMs) were fitted in R (glm; R Core Team, 2015 and glm.nb; Venables

and Ripley, 2002)) using the same abiotic explanatory variables as those above. During analysis the distribution of the residuals of each model were plotted and inspected to choose the applicable distribution. In each case the negative binomial distribution with a log link was used (Zuur et al., 2009). The model with the lowest AIC was selected using the best of both forward and backward selection procedures. As detailed above, any comparably well-fitting models within 2 AIC of the selected model were also reported. To help interpret the relationships amongst each abiotic variable and the OTU assemblage composition and richness, the deviance explained for each abiotic variable was calculated and reported.

4.5 Results and discussion

IN excess of 25 million sequences were generated from 90 samples. After filtering and clustering (at 97% similarity) the six assays produced in excess of 2000 individual Operational taxonomic units (OTUs). Of these ~ 64% could be reasonably assigned within a taxonomic framework (Table S4.3) and over half of those assigned were to a genus or species level.

Overall some contamination was noted in the eDNA samples (Tables S4.4 & S4.5): terrestrial insect (21 samples); house gecko (2 samples); dog (4 samples); gull (2 samples); and human (21 samples). Human contamination is likely the result of handling of the drop nets used for the sample collection, although the Rottnest samples were clear. Sequences that aligned most closely with insects were also found in 21 samples. While it is likely that these are the result of insect contamination, it is worth noting that they were all detected by the Copepod 3 assay. This assay targets a stretch of COI where many insect sequences are similar to some copepod sequences. The sequences best matching insects ranged from an 87–100% match, suggesting that perhaps some might really be copepods not yet found within GenBank. The insect, dog and gull contaminations are the probable result of the storage and transport of the drop nets. The nets are necessarily large (3 m long with a 0.6 m wide mouth) and unwieldy, making cleaning and protection from contamination challenging. Despite this, the overall contamination rate was minor: less than 1% of all assignable detections. The presence of contamination demonstrates the sensitivity of the metabarcoding assays used. In contrast, all DNA extraction controls were clean and all contaminant OTUs were removed prior to analysis.

4.5.1 Taxonomic biodiversity in Australian zooplankton communities

Over one thousand OTUs were assigned to over five hundred distinct taxa within the kingdom Animalia, many of which were assigned to a genus or species level (Tables S4.4 – S4.9). Table S4.10 provides details of the few other eukaryotes detected. Given the size of the data set it was not practicable to thoroughly check each assignment, an issue with large datasets that has been recognised previously (Deagle et al., 2017). However ALA (2017) revealed that ~ 76% of the assignments were known in the areas they were detected, ~ 8% were within both known and unknown areas and that ~ 90% of all assignments were known in Australia whether or not they were discovered in a known area. Figure 4.2 displays the range of taxa detected within Animalia. Within this targeted kingdom alone, almost 300 unique families were detected.

Indicator species are the species present (exclusively or in high proportions) in one site, but not others. They are identified by examining the frequency a taxon occurs at a site and its overall fidelity to that site. Indicator species were calculated using the total marine taxa detected on a presence/absence basis (*indval;* Roberts, 2016; Table S4.11; $p < 0.01$). The species identified belong to many phyla but Arthropoda and Mollusca feature strongly. Since these analyses were restricted to OTUs with assigned taxa, there may be unidentified OTUs that are more indicative of each site.

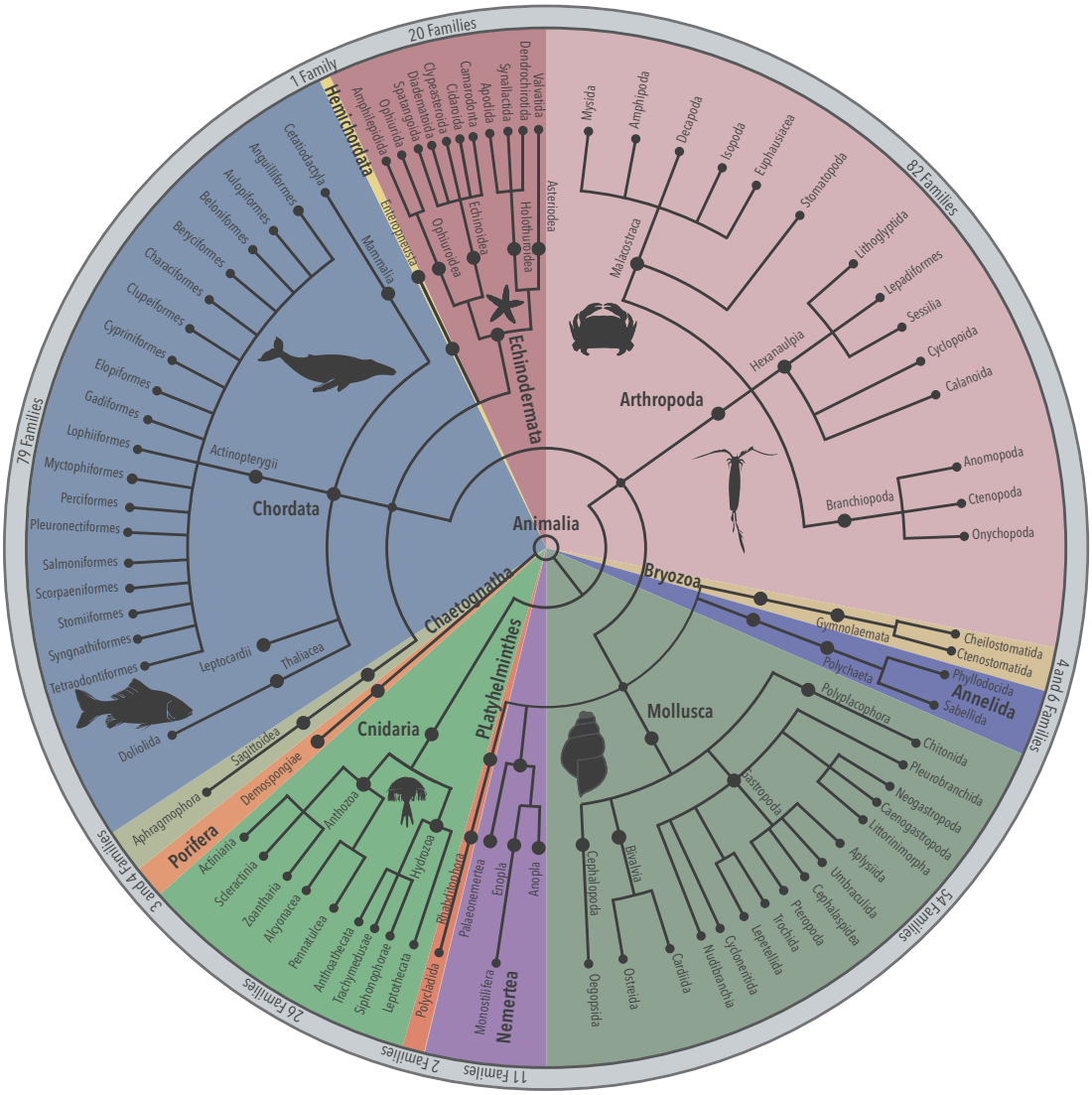

Figure 4.2: Total Animalia detected in 90 zooplankton samples using multi-gene eDNA metabarcoding. Each pie slice represents, as a proportion, the number of families detected within an individual phylum (the inner nodes). The terminal nodes represent the orders found and the mid-inner nodes the classes. The rim provides the number of families found within each phylum.

One of the more curious taxa found within the eDNA data is the detection of the iconic Humpback whale (*Megaptera novaeangliae*). While this baleen whale obviously does not have a planktonic stage to its lifecycle, it does feed on krill, small fish and plankton (Johnson and Wolman, 1984), and has a known near-coastal migration pattern, bringing it towards the IMOS sampling sites. The DNA detected is likely to have come from defecation or shed cells as the whale moved through the zooplankton or from secondary predation where detritovores, such as krill, feed upon whale waste. The whale was detected in six samples and three sites by two different assays—these detections all occurred during the whales' regular migration time and there were no detections outside of these times. One of these sites was Rottnest Island. A previous study (Berry et al. 2019—Chapter Three) used one of the same assays and the same samples at this site but did not report this taxon. An examination of unfiltered sequences from that study found that that the taxon was present in the sample but but at an abundance too low to pass the stringent filtering process. Finding whale DNA in six of the ninety samples provides evidence for the effectiveness of plankton samples as an option to detect rare species in the water column. Numerous other taxa were found that are of interest on economic, conservational and ecological grounds—a selection of them are presented in Table 4.1.

Table 4.1: A selection of commercially and environmentally important taxa detected within the plankton samples

Taxon	Site	Detections	Assays	Importance	Notes
Maoricolpus roseus (New Zealand screwshell)	Maria Is	8 of 11	Copepod 3 Mollusca	Invasive	This was the top indicator species for Maria Island, it lives in high densities and the accumulation of living and dead shells make the habitat incompatible to the survival of native species (Probst and Crawford, 2008). It was introduced to Tasmania in the 1920's (Probst and Crawford, 2008) but is now found on the east coast of Australia (ALA, 2016). The shell has a planktonic larval stage, providing a method for dispersal
Membranipora membranacea (Lacy-crust bryozoan)	Maria Is Port Hacking Kangaroo Is Esperance	5 of 11 1 of 10 8 of 8 2 of 6	Copepod 3 Mollusca Crustacea	Invasive	This was the top indicator species for Kangaroo Island. It is a colonial bryozoan that grows on the surface of kelp blades causing them to become brittle and break (Saunders and Metaxas, 2008). It causes defoliation, reduces survival of the host kelp, which then allows the infiltration of other opportunistic species (Saunders and Metaxas, 2008). While the sequences were robust matches to the reference sequences across two genes, many bryozoan species not found on the GenBank database (Benson et al., 2014—although ALA (2016) provides no alternatives)
Megaptera novaeangliae (Humpback whale)	Rottnest Is Ningaloo Port Hacking Nth Stradbroke Is	2 of 12 2 of 7 1 of 10 1 of 12	Copepod 3 16S Universal	Tourism	This whale is found all around Australia (ALA, 2016). It feeds on krill, small fish and zooplankton (Johnson and Wolman, 1984). The DNA detected is likely present in the samples from shed skin cells, saliva, urine and/or faeces. The detection times correlate with known migration patterns
Pegasus volitans (Slender sea moth)	Darwin	1 of 12	Fish Copepod 3	Potentially threatened	An IUCN report into this species stated there was insufficient data to determine its conservation status (Pollom, 2016.). It is commonly caught as by-catch from trawl fishing and sold for use in traditional Chinese medicine to replace the increasingly costly and rarer seahorse (Vincent, 2003) It is thought to be susceptible to over exploitation and has a planktonic stage in its lifecycle (Vincent, 2003)
Portunus sanguinolentus	Nth Stradbroke Is	5 of 12	Crustacea 16S Universal	Commercial	This is a commercially harvested edible crab that is found across the Indo-Pacific region (Sumpton et al., 1989).

4.5.2 Genetic diversity of zooplankton eDNA across Australia

The Australian marine environments sampled vary from tropical/equatorial (Darwin—av. SST 28.6° C) to temperate (Maria Island—av. SST 15.3° C—Figure 4.1). Accordingly the biota at each extreme is expected to be very different, as the geographical isolation of each site fosters its own system of natural selection in animals as small as zooplankton.

As expected, the specific composition of the OTUs in each sample (Assemblage—multivariate measurement) showed highly significant differences between all sites ($p \leq 0.001$—Table S4.12) and across almost all assays. The exception was the Fish assay. Fish Assemblages from Esperance and those of the southern-most sites of Port Hacking, Maria Island and Kangaroo Island were not significantly different in this study. However fish are generally larger and more mobile than the species detected by the other assays, so they have a larger geographical and depth range, than holoplankton or their more sessile meroplankton counterparts—such as Malacostraca and Mollusca. In a cross validation test (Anderson et al., 2008), samples were removed and replaced to their correct site—from their composition alone—80-90% of the time—again with the exception of the Fish assay, which had an average replacement accuracy of 56% (Table S4.13).

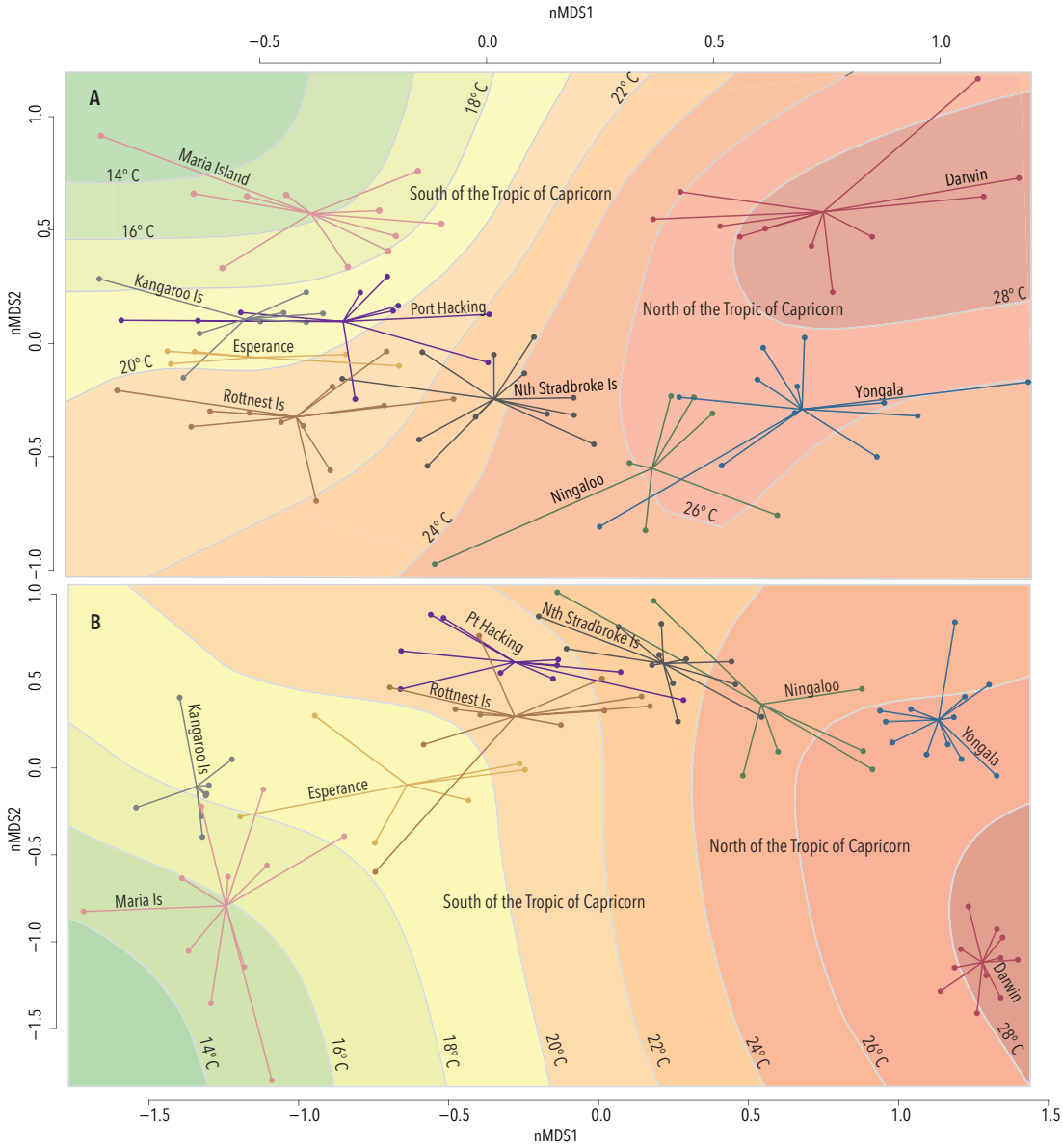

Figure 4.3: The spatial patterns evident in the eDNA planktonic Assemblages. Non-metric multidimensional scaling plots from the 16S Universal (A) and Mollusca (B) assays showing delineation in OTU assemblage between both the sampling sites and the areas north and south of the Tropic of Capricorn (stress = 0.11 & 0.10—respectively, both $k = 3$ & $p \leq 0.001$). The cross validation test—which removes and reassigns sample assemblages—produced correct assignment in 83 and 89% of the time—respectively. Clusters of the same colour represent the samples from each site and approximated sea surface temperatures are indicated. The junction of the coloured lines indicates the centroids for each site.

Figure 4.3 illustrates the site differences found within the Assemblage revealed by two of the assays (16S Universal and Mollusca). Although these assays both detect a wide range of taxa, including some fish and other meroplankton, they nevertheless exemplify the distinctions between each site and the delineation between the Assemblages from north and south of the Tropic of Capricorn.

The contours demonstrating sea surface temperature (SST) are included in Figure 4.3 to illustrate how this factor is correlated to the community composition across the sites. While the number of individual OTUs present in each sample (Richness—univariate measurement) showed less significant differences between the sites, there were still consistent responses between the southern coldest sites of Kangaroo and Maria Islands and the more northern and warmer sites (Table S4.12). Later analyses, of the interactions of abiotic factors with Richness, support this (Table 4.3) as it identifies increasing sea surface temperature (SST) as a significant positive influence on Richness.

Ninety seasonal samples, from 2012 – 2014, were subsampled from the IMOS collection. However, as some sites stopped collection of plankton in 2013, there are a total of six – twelve samples per site. The data from each assay was tested for significant seasonal differences within each site. While there were some significant seasonal changes in the Assemblage, particularly for the taxa detected by the Mollusca and Cnidaria assays, there was little significant change in Richness (Table S4.14).

Berry et al. (2019—Chapter Three) found significant seasonal responses in five years of monthly samples from Rottnest Island. The Rottnest Island data from that study was re-examined (Figure S4.1 & Table S4.15). While Richness was not overtly seasonal for the majority of assays in that study, the Fish assay results were. The results showed that the Fish assay produces significant seasonality in Richness after five samples per season (Figure S4.1).

The pairwise significance in the Assemblage increased with the number of samples. While five samples per season will provide some significant results, nine to ten samples, from each season, are required to effectively map and track seasonal changes (Table S4.15). These data help to advise how eDNA surveys might be implemented in long-term monitoring programs

SST and the concentration of silicate are the most consistent predictors for change, based on distance linear modelling (DSTLM) for the Assemblage (Table 4.2). However the closeness of the alternative models (Table S4.2) reveals that all five factors tested are significant. The concentration of salinity was included, but was absent from the most parsimonious model for all assays. The impact of the abiotic factors in the best models ranged between 11% for the Fish assay—detecting meroplankton—to 36% for the Copepod 3 assay—detecting primarily holoplankton. The implication is that obligate zooplankton are most affected by the environmental factors, at least in the short term.

SST had a positive influence on Richness, and the concentrations of phosphate and nitrate had a generally negative influence as revealed by general linear modelling (nbGLM; Venables and Ripley, 2002). The contribution of the best model varies from 3% for the Cnidaria assay to 30% for the Crustacea assay. The average variation produced by each model was 18%.

While these results, particularly with respect to SST, are not unexpected, there are limited studies linking abiotic factors to a wide variety of zooplankton (Mackas et al., 2012) and even less using multi-gene metabarcoding methods.

Assay used	OTU diversity test	Variable	SST	Silicate	Nitrate	Phosphate	Best Model	
16S Universal	Assemblage	P	<0.001	<0.001	<0.001	<0.001	R^2	0.191
		R^2	0.084	0.068	0.038	0.048		
	Richness	P	0.004	0.954			R^2	0.135
		R^2	0.088 (+)	<0.001 (-)				
Cnidaria	Assemblage	P	<0.001	<0.001		<0.001	R^2	0.243
		R^2	0.129	0.128		0.070		
	Richness	P			0.139		R^2	0.027
		R^2			0.027 (-)			
Copepod 3	Assemblage	P	<0.001	<0.001	<0.001	<0.001	R^2	0.363
		R^2	0.021	0.145	0.073	0.086		
	Richness	P	0.003			< 0.001	R^2	0.232
		R^2	0.106 (+)			0.132 (-)		
Crustacea	Assemblage	P	<0.001	<0.001	<0.001		R^2	0.148
		R^2	0.079	0.069	0.30			
	Richness	P	0.002	0.437	0.007	< 0.001	R^2	0.296
		R^2	0.109 (+)	0.008 (+)	0.084 (-)	0.138 (+)		
Fish	Assemblage	P	<0.001	<0.001	<0.001		R^2	0.109
		R^2	0.047	0.040	0.030			
	Richness	P	0.050	0.336	0.561	0.045	R^2	0.215
		R^2	0.048 (+)	0.012 (-)	0.004 (-)	0.050 (-)		
Mollusca	Assemblage	P	<0.001	<0.001	<0.001	<0.001	R^2	0.322
		R^2	0.184	0.139	0.067	0.071		
	Richness	P	< 0.001	0.009		0.273	R^2	0.202
		R^2	0.144 (+)	0.081 (+)		0.015 (-)		

Table 4.2: Models showing correlations between abiotic factors and zooplankton community response. DSTLM (Anderson et al., 2008) and nbGLM (Venables and Ripley, 2002) were used to find the most parsimonious models for OTU assemblage and richness (respectively) for the taxa detected by each assay.

4.5.3 The inherent value of spatial and temporal eDNA data

This extensive Australia-wide audit has implications for the future design of eDNA studies, whether they be spatial or temporal in nature. Table 4.3 outlines the percentage of OTUs that were found at each site for each assay and the average percentage each sample contributed towards each site's genetic diversity.

The average sample contributed between 24 – 34% to the OTUs detected at each site depending on the assay; a relatively consistent result. However the contribution of each site to the total number of OTUs detected was between 12 – 29%. It is difficult to draw conclusions from this as the results could be partly attributed to the disparity in sample number within the sites. Yet Ningaloo had a mere seven samples and was the second largest contributor of OTU diversity (28%) and Maria Island had eleven samples and was the third smallest contributor (16%).

The Southern (colder) sites are those that had the fewest OTUs when using assays that favour the larger meroplankton—Fish, Crustacea and 16S Universal. However the sites situated within large currents—the East Australian and Leeuwin—are those seemingly showing more genetic diversity both per sample and contributing to Australia wide diversity across all assays.

Given the results provided by this and the *post hoc* analysis using the extended Rottnest data (Figure S4.1 & Table S4.15), it is evident that continuous temporal and spatial samplings are needed to produce a thorough understanding of zooplankton communities. The variation in community composition within these complex populations severely limits the value of single point sampling.

Table 4.3: The total genetic diversity detected by each assay for each site and each sample within the site

Assay ➔	**16S Universal** (365 OTUs)		**Cnidaria** (285 OTUs)		**Copepod 3** (604 OTUs)		**Crustacea** (322 OTUs)		**Fish** (202 OTUs)		**Mollusca** (553 OTUs)		**Total**	
Site (samples) ➔	Site contribution to total (%)	Sample contribution to site (%)	Site contribution to total (%)	Sample contribution to site (%)	Site contribution to total (%)	Sample contribution to site (%)	Site contribution to total (%)	Sample contribution to site (%)	Site contribution to total (%)	Sample contribution to site (%)	Site contribution to total (%)	Sample contribution to site (%)	Average site contribution to total (%)	Average sample contribution to site (%)
Ningaloo (7)	17	23	26	34	40	33	20	23	24	19	41	35	28	28
Darwin (12)	23	16	28	27	23	39	22	14	16	13	32	35	24	24
Yongala (12)	20	18	22	25	27	36	19	19	24	14	22	36	22	25
Nth Stradbroke Is (12)	26	19	29	38	39	39	26	15	25	15	30	39	29	28
Port Hacking (10)	15	21	27	37	34	42	12	21	33	15	30	39	25	29
Maria Is (11)	14	21	18	30	32	20	6	24	7	20	21	28	16	24
Kangaroo Is (8)	8	31	19	34	12	45	7	31	3	27	20	36	12	34
Esperance (6)	8	26	21	35	21	39	5	33	12	21	25	37	15	32
Rottnest Is (12)	15	23	29	27	33	41	17	25	15	21	27	32	23	28
Range	8 – 26	16 – 31	18 – 29	25 – 35	12 – 40	33 – 45	6 – 26	14 – 33	3 – 33	13 – 27	20 – 41	28 – 39	12 – 29	24 – 34
Average total %	16	22	24	32	29	37	15	23	18	18	28	35	22	28

4.6 Conclusion

THIS study emphasises the power of extended spatial and temporal studies. It illustrates the ability of multi-gene metabarcoding to reveal clear spatial differences between highly complex zooplankton communities. While the sampling regime was not conducive to detecting significant seasonal changes, the combined data from Rottnest Island (Berry et al. 2019—Chapter Three) showed that two or three samples per season for five years is the starting point for subsequent eDNA surveys. This benchmarking is important to the burgeoning field of eDNA as it assists in the experimental design of eDNA based monitoring programs.

A large number of OTUs were detected and, despite database limitations, the *indval* analysis provided an indication of those taxa with distributions attributable to location. The numerous species of copepods found at each site were anticipated, but the detection of Humpback whales, was not. The other unforeseen results were the identification of two invasive species as principal indicator species at two sites. Invasive species are an ongoing environmental problem and these data reinforce the capability of eDNA to aid in ecosystem management.

These findings support the necessity for the instigation and continuation of long-term data collection and analysis using multi-gene metabarcoding. The compilation of time stamped data provides a unique resource to reveal biodiversity patterns and inform future models. Further, in what is a common refrain, as reference databases develop, presently unidentified OTUs will be assigned and so provide further understanding of zooplankton communities.

Finally, the incorporation of abiotic factors exposed their influence on the Assemblage and Richness at each site during relatively normal conditions. A continuation of this form of analysis would allow zooplankton community responses to be modelled in added detail. The ultimate goal is the integration of eDNA into more complex models of oceanic systems. While it is beyond the scope of this study, the significant interaction of eDNA with many abiotic factors suggests that the construction of models is certainly plausible.

ALA. 2016. *Atlas of Living Australia website* [Online]. http://www.ala.org.au. [Accessed 2017].

ALBERTI, A., POULAIN, J., ENGELEN, S., LABADIE, K., ROMAC, S., FERRERA, I., ALBINI, G., AURY, J.-M., BELSER, C. & BERTRAND, A. 2017. Viral to metazoan marine plankton nucleotide sequences from the Tara Oceans expedition. *Scientific data,* 4**,** sdata201793.

ALTSCHUL, S. F., GISH, W., MILLER, W., MYERS, E. W. & LIPMAN, D. J. 1990. Basic local alignment search tool. *Journal of Molecular Biology,* 215**,** 403-410.

ANDERSON, M., GORLEY, R. N. & CLARKE, R. K. 2008. *Permanova+ for Primer: Guide to Software and Statisticl Methods*, Primer-E Limited.

ANDERSON, M. J. 2001. A new method for non-parametric multivariate analysis of variance. *Austral ecology,* 26**,** 32-46.

ANDRUSZKIEWICZ, E. A., STARKS, H. A., CHAVEZ, F. P., SASSOUBRE, L. M., BLOCK, B. A. & BOEHM, A. B. 2017. Biomonitoring of marine vertebrates in Monterey Bay using eDNA metabarcoding. *PLOS ONE,* 12**,** e0176343.

BEAUGRAND, G., REID, P. C., IBANEZ, F., LINDLEY, J. A. & EDWARDS, M. 2002. Reorganization of North Atlantic marine copepod biodiversity and climate. *Science,* 296**,** 1692-4.

BENSON, D. A., CLARK, K., KARSCH-MIZRACHI, I., LIPMAN, D. J., OSTELL, J. & SAYERS, E. W. 2014. GenBank. *Nucleic Acids Research,* 42**,** D32-D37.

BERRY, T. E., OSTERRIEDER, S. K., MURRAY, D. C., COGHLAN, M. L., RICHARDSON, A. J., GREALY, A. K., STAT, M., BEJDER, L. & BUNCE, M. 2017. DNA metabarcoding for diet analysis and biodiversity: A case study

using the endangered Australian sea lion (*Neophoca cinerea*). *Ecol Evol,* 7**,** 5435-5453.

BERRY, T. E., SAUNDERS, B. J., COGHLAN, M. L., STAT, M., JARMAN, S., RICHARDSON, A. J., DAVIES, C. H., BERRY, O., HARVEY, E. S. & BUNCE, M. 2019. Marine environmental DNA biomonitoring reveals seasonal patterns in biodiversity and identifies ecosystem responses to anomalous climatic events. *PLOS Genetics,* 5(2). e1007943

BROWN, E. A., CHAIN, F. J., CREASE, T. J., MACISAAC, H. J. & CRISTESCU, M. E. 2015. Divergence thresholds and divergent biodiversity estimates: can metabarcoding reliably describe zooplankton communities? *Ecol Evol,* 5**,** 2234-51.

CASPER, R. M., JARMAN, S. N., DEAGLE, B. E., GALES, N. J. & HINDELL, M. A. 2007. Detecting prey from DNA in predator scats: A comparison with morphological analysis, using Arctocephalus seals fed a known diet. *Journal of Experimental Marine Biology and Ecology,* 347**,** 144-154.

CLARKE, K. & GORLEY, R. 2015. Getting started with PRIMER v7. *PRIMER-E: Plymouth, Plymouth Marine Laboratory*.

CLARKE, L. J., BEARD, J. M., SWADLING, K. M. & DEAGLE, B. E. 2017. Effect of marker choice and thermal cycling protocol on zooplankton DNA metabarcoding studies. *Ecol Evol,* 7**,** 873-883.

DEAGLE, B. E., CLARKE, L. J., KITCHENER, J. A., POLANOWSKI, A. M. & DAVIDSON, A. T. 2017. Genetic monitoring of open ocean biodiversity: An evaluation of DNA metabarcoding for processing continuous plankton recorder samples. *Mol Ecol Resour*.

DEAGLE, B. E., TOLLIT, D. J., JARMAN, S. N., HINDELL, M. A., TRITES, A. W. & GALES, N. J. 2005. Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions. *Mol Ecol,* 14**,** 1831-42.

EDGAR, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics,* 26**,** 2460-2461 %@ 1367-4803.

EDWARDS, M., BEAUGRAND, G., HAYS, G. C., KOSLOW, J. A. & RICHARDSON, A. J. 2010. Multi-decadal oceanic ecological datasets and their application in marine policy and management. *Trends Ecol Evol,* 25**,** 602-10.

EDWARDS, M. & RICHARDSON, A. J. 2004. Impact of climate change on marine pelagic phenology and trophic mismatch. *Nature,* 430**,** 881-884.

GATTUSO, J. P., MAGNAN, A., BILLÉ, R., CHEUNG, W. W. L., HOWES, E. L., JOOS, F., ALLEMAND, D., BOPP, L., COOLEY, S. R., EAKIN, C. M., HOEGH-GULDBERG, O., KELLY, R. P., PÖRTNER, H. O., ROGERS, A. D., BAXTER, J. M., LAFFOLEY, D., OSBORN, D., RANKOVIC, A., ROCHETTE, J., SUMAILA, U. R., TREYER, S. & TURLEY, C. 2015. Contrasting futures for ocean and society from different anthropogenic CO_2 emissions scenarios. *Science,* 349.

GIMMLER, A., KORN, R., DE VARGAS, C., AUDIC, S. & STOECK, T. 2016. The Tara Oceans voyage reveals global diversity and distribution patterns of marine planktonic ciliates. *Sci Rep,* 6**,** 33555.

GRIFFITHS, H. J., MEIJERS, A. J. S. & BRACEGIRDLE, T. J. 2017. More losers than winners in a century of future Southern Ocean seafloor warming. *Nature Climate Change,* 7**,** 749-754.

GUARDIOLA, M., URIZ, M. J., TABERLET, P., COISSAC, E., WANGENSTEEN, O. S. & TURON, X. 2015. Deep-Sea, Deep-Sequencing: Metabarcoding Extracellular DNA from Sediments of Marine Canyons. *PLoS One,* 10**,** e0139633.

HARDY, N., BERRY, T., KELAHER, B. P., GOLDSWORTHY, S. D., BUNCE, M., COLEMAN, M. A., GILLANDERS, B. M., CONNELL, S. D., BLEWITT, M. & FIGUEIRA, W. 2017. Assessing the trophic ecology of top predators

across a recolonisation frontier using DNA metabarcoding of diets. *Marine Ecology Progress Series,* 573**,** 237-254.

HARVEY, J. B. J., JOHNSON, S. B., FISHER, J. L., PETERSON, W. T. & VRIJENHOEK, R. C. 2017. Comparison of morphological and next generation DNA sequencing methods for assessing zooplankton assemblages. *Journal of Experimental Marine Biology and Ecology,* 487**,** 113-126.

HERON, A. C. 1982. A vertical free fall plankton net with no mouth obstructions. *Limnology and Oceanography,* 27**,** 380-383.

HUSON, D. H., MITRA, S., RUSCHEWEYH, H. J., WEBER, N. & SCHUSTER, S. C. 2011. Integrative analysis of environmental sequences using MEGAN 4, . *Genome Res.,* 21**,** 1552-1560.

JOHNSON, J. H. & WOLMAN, A. A. 1984. The humpback whale, Megaptera novaeangliae. *Marine Fisheries Review,* 46**,** 30-37.

KEARSE, M., MOIR, R., WILSON, A., STONES-HAVAS, S., CHEUNG, M., STURROCK, S., BUXTON, S., COOPER, A., MARKOWITZ, S., DURAN, C., THIERER, T., ASHTON, B., MEINTJES, P. & DRUMMOND, A. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics,* 28**,** 1647-1649.

LENANTON, R. C. J., DOWLING, C. E., SMITH, K. A., FAIRCLOUGH, D. V. & JACKSON, G. 2017. Potential influence of a marine heatwave on range extensions of tropical fishes in the eastern Indian Ocean—Invaluable contributions from amateur observers. *Regional Studies in Marine Science,* 13**,** 19-31.

LINDEQUE, P.K., PARRY, H.E., HARMER, R.A., SOMERFIELD, P.J. & ATKINSON, A. 2013. Next generationn sequencing reveals the hidden diversity of zooplankton assemblages. *Plos One,* 8, 11, e81327

LOTZE, H. K., LENIHAN, H. S., BOURQUE, B. J., BRADBURY, R. H., COOKE, R. G., KAY, M. C., KIDWELL, S. M., KIRBY, M. X., PETERSON, C. H. & JACKSON, J. B. C. 2006. Depletion, Degradation, and Recovery Potential of Estuaries and Coastal Seas. *Science,* 312**,** 1806.

LYNCH, T. P., MORELLO, E. B., EVANS, K., RICHARDSON, A. J., ROCHESTER, W., STEINBERG, C. R., ROUGHAN, M., THOMPSON, P., MIDDLETON, J. F., FENG, M., SHERRINGTON, R., BRANDO, V., TILBROOK, B., RIDGWAY, K., ALLEN, S., DOHERTY, P., HILL, K. & MOLTMANN, T. C. 2014. IMOS National Reference Stations: a continental-wide physical, chemical and biological coastal observing system. *PLoS One,* 9**,** e113652.

MACKAS, D. L., GREVE, W., EDWARDS, M., CHIBA, S., TADOKORO, K., ELOIRE, D., MAZZOCCHI, M. G., BATTEN, S., RICHARDSON, A. J., JOHNSON, C., HEAD, E., CONVERSI, A. & PELUSO, T. 2012. Changing zooplankton seasonality in a changing ocean: Comparing time series of zooplankton phenology. *Progress in Oceanography,* 97-100**,** 31-62.

MCCAULEY, D. J., PINSKY, M. L., PALUMBI, S. R., ESTES, J. A., JOYCE, F. H. & WARNER, R. R. 2015. Marine defaunation: animal loss in the global ocean. *Science,* 347**,** 1255641.

MORARD, R., LEJZEROWICZ, F., DARLING, K. F., LECROQ-BENNET, B., PEDERSEN, M. W., ORLANDO, L., PAWLOWSKI, J., MULITZA, S., DE VARGAS, C. & KUCERA, M. 2017. Planktonic foraminifera-derived environmental DNA extracted from abyssal sediments preserves patterns of plankton macroecology. *Biogeosciences,* 14**,** 2741.

OKSANEN, J., GUILLAUME BLANCHET, F., FRIENDLY, M., KINDT, R., LEGENDRE, P., MCGLINN, D., MINCHIN, P. R., O'HARA, R. B., SIMPSON, G. L., SOLYMOS, P., STEVENS, M. H. H., SZOECS, E. & WAGNER, H. 2016. vegan: Community Ecology Package. R package version 2.3-0. http://CRAN.R-project.org/package=vegan.

POLLOM, R. 2016. . *Pegasus volitans* (errata version published in 2017). The IUCN Red List of Threatened Species 2016: e.T16476A115133968.

PROBST, T. A. & CRAWFORD, C. M. 2008. Population characteristics and planktonic larval stage of the New Zealand screwshell *Maoricolpus roseus*. *Journal of Molluscan Studies,* 74**,** 191-197.

R CORE TEAM. 2015. *R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria* [Online]. Vienna, Austria. Available: http://www.R-project.org/. [Accessed 2016].

RICHARDSON, A. 2009. Plankton and climate. *Elements of Physical Oceanography: A derivative of the Encyclopedia of Ocean Sciences***,** 397.

RICHARDSON, A. J., BROWN, C. J., BRANDER, K., BRUNO, J. F., BUCKLEY, L., BURROWS, M. T., DUARTE, C. M., HALPERN, B. S., HOEGH-GULDBERG, O., HOLDING, J., KAPPEL, C. V., KIESSLING, W., MOORE, P. J., O'CONNOR, M. I., PANDOLFI, J. M., PARMESAN, C., SCHOEMAN, D. S., SCHWING, F., SYDEMAN, W. J. & POLOCZANSKA, E. S. 2012. Climate change and marine life. *Biol Lett,* 8**,** 907-9.

ROBERTS, D. W. 2016. labdsv: Ordination and Multivariate Analysis for Ecology. R package version 1.8-0.

SAUNDERS, M. & METAXAS, A. 2008. High recruitment of the introduced bryozoan Membranipora membranacea is associated with kelp bed defoliation in Nova Scotia, Canada. *Marine Ecology Progress Series,* 369**,** 139-151.

STAT, M., HUGGETT, M. J., BERNASCONI, R., DIBATTISTA, J. D., BERRY, T. E., NEWMAN, S. J., HARVEY, E. S. & BUNCE, M. 2017. Ecosystem biomonitoring with eDNA: metabarcoding across the tree of life in a tropical marine environment. *Sci Rep,* 7**,** 12240.

SUMPTON, W. D., SMITH, G. S. & POTTER, M. A. 1989. Notes on the Biology of the Portunid Crab, *Portunus sanguinolentus* (Herbst), in Subtropical Queensland Waters. *Marine and Freshwater Research,* 40**,** 711-717.

TABERLET, P., BONIN, A., ZINGER, L. & COISSAC, E. 2018. Environmental DNA for biodiversity research and monitoring. *Analysis of bulk samples.* Oxford University Press. Chapter 18

VENABLES, W. & RIPLEY, B. 2002. Random and mixed effects. *Modern applied statistics with S.* Springer.

VINCENT AMANDA, C. J. 2003. Trade in pegasid fishes (sea moths), primarily for traditional Chinese medicine. *Oryx,* 31**,** 199-208.

WERNBERG, T., BENNETT, S., BABCOCK, R. C., DE BETTIGNIES, T., CURE, K., DEPCZYNSKI, M., DUFOIS, F., FROMONT, J., FULTON, C. J., HOVEY, R. K., HARVEY, E. S., HOLMES, T. H., KENDRICK, G. A., RADFORD, B., SANTANA-GARCON, J., SAUNDERS, B. J., SMALE, D. A., THOMSEN, M. S., TUCKETT, C. A., TUYA, F., VANDERKLIFT, M. A. & WILSON, S. 2016. Climate-driven regime shift of a temperate marine ecosystem. *Science,* 353**,** 169.

ZUUR, A., IENO, E., WALKER, N., SAVELIEV, A. & SMITH, G. 2009. Mixed effects models and extensions in ecology with R. New York: Springer. *574 p*.

Table S4.1: Details of the PCR assays used within this study, and their annealing temperatures

PCR assay	Primer set used	Primary target taxa	Gene	Primer sequence	Amplicon length (bp)	Reference	Assay Ta (°C)
16S Universal	16s1F -degenerate 16s2R - degenerate	Universal	16S rRNA	5′ GACGAKAAGACCCTA 3′ 5′ CGCTGTTATCCCTADRGTAACT 3′	180-270 bp	Deagle et al. (2007)	54 °C
Cnidaria	Cnidaria_F Cnidaria_R	Cnidarians	COI	5′ CATGATHTTYTTCWTDGTMATGCC 3′ 5′ GTYCAWCCWGTWCCWRCYCC 3′	~145 bp	Berry, et al. (2019)	52 °C
Copepod 3	Acartia_F Acartia_R	Copepods	COI	5′ GGRGAYGATCARRTYTATAAYGT 3′ 5′ TTYATWCGWGGAAAHGCYATRTC 3′	~103 bp	Berry, et al. (2019)	50 °C
Crustacea	Crust16S_ F (short) Crust16S_ R (short)	Crustaceans	16S rRNA	5′ GGGACGATAAGACCCTATA 3′ 5′ ATTACGCTGTTATCCCTAAAG 3′	~170 bp	Berry et al. (2017)	51 °C
Mollusca	Limacina_F Limacina_R	Molluscs	COI	5′ TAATTGGNGGVTTTGGRAAYTG 3′ 5′ GTTCAHCCTRAYCCTRCNCC 3′	~118 bp	Berry, et al. (2019)	52 °C
Fish	16s2R - degenerate Fish16sF/D	Actinopterygii	16S rRNA	5′ CGCTGTTATCCCTADRGTAACT 3′ 5′ GACCCTATGGAGCTTTAGAC 3′	~200 bp	F- Deagle et al. (2007) R- Berry et al. (2017)	54 °C

'F' refers to the forward primer; 'R' refers to the reverse primer.

Table S4.2: Alternative models showing the influence of abiotic factors on Richness and Assemblage within two AIC of the most parsimonious models—DSTLM (Anderson et al., 2008) & nbGLM (Venables and Ripley, 2002)

Assay used	OTU diversity test	Most parsimonious model (R^2)	Alternative Model 1	Alternative Model 2	Alternative Model 3	Alternative Model 4
16S Universal	Assemblage	SST, Silicate, Nitrate, Phosphate (0.191)	SST, Silicate, Nitrate	SST, Salinity, Silicate, Nitrate, Phosphate	SST, Silicate	
	AIC	651.32	651.62	652.05	652.84	
	Richness	SST, Silicate (0.135)	SST, Nitrate, Silicate			
	AIC	484.59	486.45			
Cnidaria	Assemblage	SST, Silicate, Phosphate (0.243)	SST, Silicate, Nitrate, Phosphate	SST, Salinity, Silicate, Nitrate, Phosphate	SST, Silicate	
	AIC	606.47	606.65	607.11	607.54	
	Richness	Nitrate (0.027)	Nitrate, Phosphate			
	AIC	562.86	564.21			
Copepod3	Assemblage	SST, Silicate, Nitrate, Phosphate (0.363)	SST, Salinity, Silicate, Nitrate, Phosphate	SST, Silicate, Phosphate		
	AIC	580.84	581.75	581.85		
	Richness	SST, Phosphate (0.232)	SST, Nitrate, Phosphate	SST, Nitrate, Silicate, Phosphate	SST, Nitrate, Salinity, Silicate, Phosphate	
	AIC	693.37	693.71	694.15	694.60	
Crustacea	Assemblage	SST, Silicate, Nitrate (0.148)	SST, Silicate, Nitrate, Phosphate	SST, Silicate	SST, Nitrate, Salinity, Silicate, Phosphate	
	AIC	654.44	654.57	655.25	655.56	
	Richness	SST, Nitrate, Silicate, Phosphate (0.296)	SST, Nitrate, Phosphate	SST, Phosphate	SST, Nitrate, Salinity, Silicate, Phosphate	
	AIC	470.53	470.65	470.93	471.03	
Fish	Assemblage	SST, Silicate, Nitrate (0.109)	SST, Nitrate	SST, Silicate, Nitrate, Phosphate	SST	SST, Nitrate, Salinity, Silicate, Phosphate
	AIC	619.19	619.28	619.59	620.19	620.76
	Richness	SST, Nitrate, Silicate, Phosphate (0.215)	SST, Phosphate, Nitrate	SST, Salinity, Nitrate, Silicate, Phosphate		
	AIC	396.46	398.03	398.34		
Mollusca	Assemblage	SST, Silicate, Nitrate, Phosphate (0.322)	SST, Silicate, Phosphate	SST, Salinity, Silicate, Nitrate, Phosphate		
	AIC	599.13	599.64	599.99		
	Richness	SST, Silicate, Phosphate (0.202)	Phosphate, SST	SST, Salinity, Silicate, Phosphate	SST	
	AIC	661.4	662.13	662.67	662.86	

Table S4.3: Sequence numbers produced, the number of operational taxonomic units (OTUs) created and the percentage of the OTUs assigning to taxa, for each assay

Assay	Approx. length of barcode (bp)	Unfiltered sequence numbers	Number of OTUs	Approx. % taxonomically assigned
16S Universal	140-210	387,040	370	44
Cnidaria	145	5,283,966	287	54
Copepod 3	103	7,678,955	614	83
Crustacea	130-180	876,295	120	37
Fish	200-220	5,275,617	202	77
Mollusca	120	5,743,736	556	51
Totals		25,245,609	2149	64

Table S4.4: The number of Arthropoda detections within the Australian zooplankton samples—match and location determined using GenBank (Benson et al. 2014) and ALA (2016) respectively.

Class Order	**Family**	**Genus**	**Species**	**Area**	**Australia**	**% Match**	**Assay**	**ESP**	**MAI**	**NIN**	**DAR**	**KI**	**NSI**	**PH**	**YON**	**ROT**
Arthropoda (phyla)				Y		85-97%	Cr, M, 16S, C3	31	28	88	129	38	99	71	113	116
Branchiopoda				Y		86-93%	C3	0	0	4	0	0	7	0	2	1
Anomopoda				M		89-90%	C3	1	1	2	0	1	0	0	0	0
Ctenopoda	Sididae	*Penilia*	*Penilia avirostris*	Y		99-100%	M, C3	6	2	0	0	6	3	9	1	9
Onychopoda	Podonidae			Y		92%	16S	1	0	0	0	1	0	5	0	0
		Evadne		Y		96-98%	16S, C3	2	3	0	0	1	0	4	0	6
			Evadne nordmanni	Y		98-100%	M, Cn, C3	0	7	0	0	0	0	2	0	0
			Evadne spinifera	Y		98-100% (v)	M, 16S, Cn	3	1	0	0	4	0	4	0	5
		Podon	*Podon intermedius*	Y		99-100%	Cr, 16S	0	6	0	0	2	0	4	0	0
		Pseudevadne		Y		95-96%	M, 16S	3	0	0	0	1	0	0	0	2
			Pseudevadne tergestina	Y		98-100%	M, Cn, C3	3	0	1	0	1	3	10	3	0
Insecta				Y		95-97%	C3	2	2	0	3	4	0	0	2	8
Hexanauplia				Y		87-100%	M, 16S, Cn, C3	9	25	14	20	16	41	34	14	19
Calanoida				Y		89-100%	M, Cn, C3	23	20	43	73	19	70	65	46	84
	Acartiidae			Y		94%	C3	0	0	0	0	0	1	0	1	1
		Acartia		Y		95-98% (v)	C3	0	0	1	0	0	0	0	5	1
			Acartia (Acartia) negligens	Y		98-100% (v)	C3, Cn	3	1	7	0	1	12	10	8	11
			Acartia (Acartia) danae	Y		99-100% (v)	M, C3	4	8	4	0	8	9	10	1	10
			Acartia (Odontacartia) erythraea	Y		99% (v)	Cn	0	0	2	0	0	2	1	2	0
	Augaptilidae	*Haloptilus*	*Haloptilus longicornis*	N	Y	100% (v)	C3	0	0	0	0	0	3	8	0	0
	Calanidae			Y		92-93%	C3	0	0	1	0	0	12	11	2	4
		Calanus		Y		96%	Cn	0	12	0	0	1	0	0	0	0
			Calanus Australis	M		98-100% (v)	M, Cn, C3	3	11	2	0	3	0	2	0	0
		Canthocalanus	*Canthocalanus pauper*	Y		98-100%	M, C3	1	0	6	5	0	12	8	12	4
		Cosmocalanus		Y		96%	C3	1	0	3	0	0	6	5	0	5
			Cosmocalanus darwinii	Y		100%	C3	1	0	6	0	0	11	9	2	7
		Mesocalanus		Y		95%	C3	0	0	0	0	0	0	2	0	0

Class Order	**Family**	**Genus**	**Species**	**Area**	**Australia**	**% Match**	**Assay**	**ESP**	**MAI**	**NIN**	**DAR**	**KI**	**NSI**	**PH**	**YON**	**ROT**
		Neocalanus		Y		96-97%	M, C3	0	3	0	0	0	0	0	0	0
			Neocalanus gracilis	Y		99% (v)	M	0	0	1	0	0	0	0	0	0
			Neocalanus tonsus	Y		98-100% (v)	M, C3	2	8	2	0	0	0	0	0	0
		Undinula	*Undinula vulgaris*	Y		98-100% (v)	M, C3	2	0	6	0	0	11	4	11	6
	Candaciidae	*Candacia*		Y		96%	16S, C3	2	4	0	5	3	0	6	2	8
			Candacia bradyi	M		99%	M	5	0	1	10	0	6	1	5	9
			Candacia discaudata	M		100% (v)	M	0	0	5	0	0	8	1	11	1
			Candacia simplex	N	Y	99% (v)	C3	0	0	0	0	1	4	3	0	1
			Candacia truncata	M		100% (v)	C3	1	0	6	0	0	9	5	0	4
	Centropagidae	*Boeckella*		M		95%	M	1	0	4	0	0	9	8	2	5
		Centropages		Y		97%	16S	0	2	0	0	0	0	2	0	0
			Centropages furcatus	Y		98-99% (v)	C3	0	0	8	10	0	23	15	23	4
			Centropages orsinii	M		98-99%	M, C3	0	0	4	2	0	10	7	8	3
	Clausocalanidae			Y		92-93%	C3	1	4	0	0	3	6	7	0	8
		Clausocalanus		Y		96-100%	C3	0	5	0	0	3	2	2	0	2
			Clausocalanus arcuicornis	Y		100%	C3	1	4	3	0	4	7	6	0	8
			Clausocalanus furcatus	Y		98-100% (v)	M, C3	17	10	13	0	15	35	30	15	35
			Clausocalanus ingens	M		100% (v)	M, C3	2	9	2	0	0	0	2	0	1
			Clausocalanus jobei	Y		99-100% (v)	C3	4	3	0	0	4	1	10	0	5
			Clausocalanus lividus	M		99-100%	C3	0	0	0	0	0	3	4	0	0
			Clausocalanus minor	M		99%	C3	2	0	7	0	0	11	9	8	9
			Clausocalanus paululus	M		98-99% (v)	M	0	3	3	0	2	6	8	0	7
			Clausocalanus pergens	Y		99-100% (v)	C3	1	9	0	0	4	2	10	0	6
		Ctenocalanus	*Ctenocalanus vanus*	Y		98-100% (v)	M, C3	4	9	2	0	8	10	10	0	10
	Eucalanidae			Y		95%	C3	0	0	0	0	0	0	0	0	1
		Pareucalanus	*Pareucalanus sewelli*	N	N	100% (v)	C3	0	0	2	0	0	4	1	0	1
	Euchaetidae	*Euchaeta*	*Euchaeta longicornis*	N	N	100% (v)	Cr	0	0	1	0	0	0	0	0	2
	Lucicutiidae			Y		92-93%	M, C3	1	2	1	0	0	5	7	0	4
		Lucicutia		Y		96%	Cr	0	0	0	0	0	2	1	0	0
			Lucicutia flavicornis	Y		100% (v)	M	0	1	5	0	3	12	12	0	10
	Metridinidae			Y		93%	16S	0	3	0	0	0	0	0	0	0
		Pleuromamma		Y		98-99%	16S, C3	0	2	0	0	0	1	0	0	0

Class Order	**Family**	**Genus**	**Species**	**Area**	**Australia**	**% Match**	**Assay**	**ESP**	**MAI**	**NIN**	**DAR**	**KI**	**NSI**	**PH**	**YON**	**ROT**
			Pleuromamma abdominalis	Y		98-100% (v)	M, C3	0	2	0	0	0	2	5	0	0
			Pleuromamma borealis	Y		98-100% (v)	M, 16S	0	2	0	0	0	0	0	0	0
	Paracalanidae			Y		92-94%	M, C3	4	0	12	24	1	15	8	3	16
		Bestiolina		Y		95-100%	C3, M	6	1	6	25	7	6	11	1	11
		Calocalanus		Y		95-97%	M, C3	5	6	7	0	1	11	14	1	16
			Calocalanus minutus	N	N	100% (v)	C3	4	0	7	0	1	9	5	0	8
			Calocalanus pavo	Y		98-100% (v)	M, C3	3	0	5	0	3	10	6	4	8
			Calocalanus plumulosus	Y		100% (v)	C3	2	0	5	0	0	10	7	1	6
			Calocalanus styliremis	Y		98% (v)	C3	1	1	6	0	1	7	4	0	6
			Calocalanus tenuis	N	N	98-99% (v)	M, C3	0	8	0	0	1	0	0	0	0
		Delibus		N	N	96-100%	M, C3	6	4	15	3	0	25	19	12	19
		Mecynocera	*Mecynocera clausi*	Y		97-100% (v)	M	4	2	6	0	13	17	14	1	19
		Paracalanus		Y		95-100% (v)	M, C3	9	17	19	1	8	46	57	30	37
			Paracalanus aculeatus	M		100%	C3	2	0	7	11	0	12	6	12	7
			Paracalanus indicus	Y		98-100% (v)	M, C3	8	8	8	0	6	7	5	6	14
			Paracalanus nanus	N	N	98-100% (v)	M, 16S, C3	5	3	5	0	6	8	8	1	11
			Paracalanus tropicus	N	N	99-100%	C3	4	1	5	0	1	11	10	4	11
		Parvocalanus	*Parvocalanus crassirostris*	Y		98-100%	M, C3	0	0	0	12	0	0	0	0	0
	Pontellidae	*Labidocera*	*Labidocera acuta*	M		99-100% (v)	M, Cn, C3	0	0	3	3	1	12	9	12	2
			Labidocera minuta	M		100%	M, C3	7	0	4	1	2	12	8	12	11
		Pontellina	*Pontellina plumata*	M		98-100% (v)	Cn, C3	1	0	2	0	0	2	3	0	4
	Scolecitrichidae	*Scolecithricella*	*Scolecithricella longispinosa*	N	N	100% (v)	M, C3	0	0	2	5	0	7	1	1	0
		Scolecithrix	*Scolecithrix danae*	Y		100% (v)	C3	1	0	4	0	0	7	9	0	6
	Subeucalanidae	*Subeucalanus*		Y		100% (v)	M, C3	1	0	3	8	0	8	5	11	5
			Subeucalanus mucronatus	Y		100% (v)	M, Cn, C3	0	0	3	0	0	6	4	0	0
			Subeucalanus pileatus	Y		99-100% (v)	M, C3	0	0	3	0	0	10	8	4	4
			Subeucalanus subtenuis	N	N	100% (v)	C3	2	0	2	0	1	0	0	0	8
	Temoridae			Y		93%	C3	0	5	0	0	0	0	2	0	0
		Temora	*Temora discaudata*	M		100% (v)	M, C3	2	1	1	0	0	9	9	4	6
	Tortanidae			Y		94%	M	0	0	0	5	0	0	0	0	0
Cyclopoida				Y		89-91%	M, Cn	0	0	4	0	0	7	2	0	0
	Corycaeidae			Y		94%	M	5	3	5	0	8	1	3	6	10

Class Order	**Family**	**Genus**	**Species**	**Area**	**Australia**	**% Match**	**Assay**	**ESP**	**MAI**	**NIN**	**DAR**	**KI**	**NSI**	**PH**	**YON**	**ROT**
		Ditrichocorycaeus		Y		95-96%	C3, M	5	2	3	10	5	8	3	14	12
		Farranula	*Farranula gibbula*	Y		98-100% (v)	M, C3	0	0	6	0	0	8	5	4	3
	Oithonidae			Y		100%	M	2	0	3	0	0	4	2	0	5
	Oncaeidae			Y		89-90%	M	1	0	5	1	0	2	3	3	2
		Oncaea	*Oncaea media*	Y		98% (v)	Cn	1	0	4	0	0	3	2	1	4
			Oncaea mediterranea	Y		99-100% (v)	M, C3	0	0	4	0	0	5	3	0	3
			Oncaea scottodicarloi	N	Y	98%	M	0	0	0	0	1	0	0	0	0
	Sapphirinidae	*Copilia*	*Copilia mirabilis*	Y		100% (v)	M	0	0	1	0	0	7	2	3	0
		Sapphirina	*Sapphirina angusta*	M		99% (v)	M	0	1	0	0	0	1	2	0	0
Lepadiformes	Lepadidae	*Conchoderma*	*Conchoderma virgatum*	N	Y	100%	Cn	0	0	1	0	0	0	0	0	2
Lithoglyptida	Lithoglyptidae	*Kochlorine*		N	Y	97%	C3	0	0	3	0	0	0	0	0	2
Sessilia				Y		90%	M	0	0	0	1	0	0	0	0	0
	Balanidae	*Amphibalanus*		Y		95%	M	0	0	0	2	0	0	0	0	0
	Tetraclitidae	*Tetraclitella*	*Tetraclitella purpurascens*	Y		99-100%	M, 16S	0	1	0	0	0	0	1	0	0
Malacostraca				Y		86-94%	Cr, M, 16S, C3	3	6	9	10	8	11	8	15	20
Amphipoda				Y		90-94%	M, C3	0	2	1	1	2	4	0	0	1
	Lestrigonidae	*Hyperietta*		Y		97%	C3	0	0	1	0	0	0	0	0	0
			Hyperietta parviceps	N	N	98%	M	0	0	1	0	0	0	0	0	0
		Hyperioides	*Hyperioides sibaginis*	S		99-100% (v)	C3	0	0	3	0	0	7	4	2	1
Decapoda				Y		89-100%	Cr, M, 16S, Cn, C3	8	4	27	36	7	19	4	24	33
	Alpheidae			Y		92%	M, C3	2	0	2	0	0	1	0	0	4
		Metalpheus	*Metalpheus paragracilis*	Y		100%	Cr, 16S	0	0	4	0	0	0	0	0	0
		Synalpheus		Y		95-96%	Cr, 16S	0	0	0	0	0	0	0	1	0
	Calappidae			Y		92-93%	Cr	0	0	0	0	0	1	0	0	0
		Calappa	*Calappa gallus*	Y		98% (v)	Cr	0	0	2	0	0	0	0	0	0
	Callianassidae			Y		94%	M	0	0	0	1	0	0	0	0	0
		Trypaea	*Trypaea australiensis*	Y		100%	Cr	0	0	0	0	0	1	0	0	0
	Corystoidea	*Jonas*		Y		95%	Cr	0	0	0	0	0	1	0	2	0
	Crangonidae			Y		92%	C3	0	1	1	0	5	2	3	0	4
		Crangon		N	Y	97%	Cr	2	0	0	0	0	0	0	0	0
	Diogenidae	*Calcinus*	*Calcinus dapsiles*	Y		100%	Cr, 16S, C3	0	0	0	0	0	0	0	0	3
			Calcinus vachoni	N	N	99%	Cr, C3	0	0	1	0	0	0	0	0	0

Class Order	**Family**	**Genus**	**Species**	**Area**	**Australia**	**% Match**	**Assay**	**ESP**	**MAI**	**NIN**	**DAR**	**KI**	**NSI**	**PH**	**YON**	**ROT**
		Ciliopagurus	*Ciliopagurus strigatus*	N	Y	100% (v)	C3	0	0	1	0	0	0	0	0	0
		Dardanus	*Dardanus arrosor*	Y		98-100% (v)	Cr, 16S, C3	0	4	0	0	0	0	1	0	0
	Epialtidae	*Menaethius*		N	Y	95%	Cr	0	0	0	0	0	0	0	0	1
			Menaethius monoceros	Y		99-100%	Cr, 16S	0	0	0	1	0	0	0	0	0
	Galatheidae			Y		92-99%	Cr, C3	0	0	3	1	0	0	0	1	0
		Galathea		Y		98-100% (v)	Cr	0	0	0	0	0	1	0	1	0
	Goneplacidae			Y		94%	Cr	0	1	0	0	0	0	0	0	0
		Carcinoplax		Y		96%	16S	0	1	0	0	0	0	0	0	0
		Pycnoplax		Y		96%	Cr	0	1	0	0	0	0	0	0	0
	Grapsoidea	*Metopograpsus*		Y		96-97%	Cr, 16S	0	0	0	1	0	0	0	0	0
			Metopograpsus frontalis	Y		100% (v)	Cr	0	0	0	1	0	0	0	0	0
	Hippolytidae	*Alope*	*Alope orientalis*	Y		99%	Cr	0	0	0	0	0	0	1	0	0
		Latreutes	*Latreutes pymoeus*	N	Y	100%	Cr	0	0	0	0	0	0	0	3	0
	Luciferidae	*Belzebub*	*Belzebub intermedius*	N	N	99-100%	C3	3	0	2	0	0	7	1	7	5
		Lucifer		M		95-100%	M, C3	0	0	3	6	0	4	2	5	1
	Lysmatidae	*Lysmata*	*Lysmata ternatensis*	N	N	100% (v)	Cr	0	0	2	0	0	0	0	0	0
	Macrophthalmidae			Y		93%	Cr, 16S	0	0	0	1	0	2	0	1	0
		Macrophthalmus		Y		95-96%	Cr, 16S, C3	0	0	0	1	0	0	0	7	0
	Matutidae			Y		93%	Cr	0	0	0	2	0	0	0	0	0
	Mictyridae	*Mictyris*	*Mictyris longicarpus*	Y		100%	Cr	0	0	0	1	0	0	0	0	0
	Ocypodidae			Y		93-94%	Cr, 16S, C3	0	0	0	5	0	0	0	3	0
		Tubuca	*Tubuca signata*	N	N	100%	Cr, 16S	0	0	0	1	0	0	0	0	0
	Ovalipidae	*Ovalipes*		Y		95-97%	Cr	0	0	0	0	1	0	0	0	0
	Paguridae			Y		93%	C3	0	0	0	0	0	1	0	0	0
	Palaemonidae	*Rapimenes*	*Rapimenes laevimanus*	N	N	98% (v)	Cr	0	0	1	0	0	0	0	0	0
	Pandalidae			Y		92-94%	Cr, M, 16S	0	0	0	0	0	1	1	4	0
		Chlorotocus		Y		95-96%	Cr, 16S	0	1	0	0	0	0	1	0	0
	Parthenopidae			Y		93%	M	0	0	0	1	0	0	0	0	0
	Pasiphaeidae			Y		94%	Cr	0	0	2	0	0	0	0	0	1
		Leptochela		Y		95-96%	Cr, 16S	0	4	0	0	2	3	4	4	1
	Penaeidae			Y		92-94%	M, 16S, C3	0	0	0	7	0	1	0	0	0
		Atypopenaeus	*Atypopenaeus stenodactylus*	Y		99%	Cr, 16S	0	0	0	3	0	0	0	0	0

Class Order	**Family**	**Genus**	**Species**	**Area**	**Australia**	**% Match**	**Assay**	**ESP**	**MAI**	**NIN**	**DAR**	**KI**	**NSI**	**PH**	**YON**	**ROT**
		Metapenaeopsis		Y		95-97%	Cr, M, 16S	0	0	0	5	0	0	0	3	0
			Metapenaeopsis lamellata	Y		98%	Cr	0	0	0	0	0	0	0	1	0
			Metapenaeopsis palmensis	Y		98-99%	Cr, 16S	0	0	0	2	0	0	0	2	0
			Metapenaeus monoceros	N	N	99%	C3	0	0	0	0	0	1	0	0	0
		Penaeus	*Penaeus latisulcatus*	Y		99%	Cr, 16S	0	0	2	0	1	0	0	2	0
			Penaeus plebejus	Y		100%	Cr	0	0	0	0	0	2	0	0	0
		Trachypenaeus	*Trachypenaeus anchoralis*	Y		100% (v)	Cr, 16S	0	0	0	2	0	0	0	0	0
		Trachysalambria	*Trachysalambria malaiana*	N	Y	99% (v)	Cr, 16S	0	0	0	2	0	0	0	0	0
			Trachysalambria nansei	N	Y	99% (v)	Cr	0	0	0	0	0	4	0	2	0
	Pinnotheridae	*Tetrias*	*Tetrias fischerii*	N	N	100% (v)	Cr	0	0	0	0	0	2	0	0	0
	Polybiidae	*Liocarcinus*	*Liocarcinus corrugatus*	M		98% (v)	Cr, 16S	0	2	0	0	2	0	0	0	1
	Porcellanidae	*Petrolisthes*	*Petrolisthes haswelli*	Y		100%	Cr, C3	0	0	0	1	0	0	0	0	0
	Portunidae			Y		92-93%	C3	0	1	1	0	0	2	0	3	0
		Charybdis	*Charybdis anisodon*	Y		99%	Cr	0	0	0	1	0	0	0	0	0
		Libystes	*Libystes edwardsi*	Y		100% (v)	Cr	0	0	0	0	0	1	0	0	0
		Portunus	*Portunus sanguinolentus*	Y		99-100% (v)	Cr, 16S	0	0	0	0	0	5	0	0	0
			Portunus (Xiphonectes) tenuipes	Y		99%	C3	0	0	0	0	0	0	0	3	0
		Thalamita		Y		95%	C3	0	0	0	1	0	0	0	0	0
			Thalamita admete	Y		98-100% (v)	Cr, 16S	0	0	5	1	0	1	0	1	0
	Processidae	*Processa*		Y		97%	Cr	0	0	0	0	0	0	0	1	0
	Scyllaridae			Y		94%	C3	0	0	0	4	0	0	0	0	0
		Petrarctus		Y		98%	Cr, 16S	0	0	1	0	0	0	0	0	0
	Sergestidae			Y		93%	C3	0	0	0	0	0	0	0	3	0
	Sesarmidae			Y		92-96%	Cr, C3	0	0	0	4	0	0	0	0	0
		Neosarmatium	*Neosarmatium meinerti*	Y		98-99%	C3	0	0	0	1	0	0	0	0	0
	Trapeziidae			N	Y	92%	M	1	0	0	0	0	0	0	0	5
	Upogebiidae	*Gebiacantha*	*Gebiacantha plantae*	N	N	100% (v)	Cr, 16S	0	0	0	0	0	0	0	5	0
	Varunidae	*Paragrapsus*	*Paragrapsus laevis*	Y		99-100%	16S	0	1	0	0	0	0	0	0	0
	Xanthidae			Y		92-93%	Cr, 16S	0	0	0	0	2	1	1	0	0
		Actaea		Y		95%	Cr	0	0	0	0	0	0	0	0	1
		Euxanthus	*Euxanthus ruali*	N	N	99% (v)	Cr	0	0	1	0	0	0	0	0	0

Class Order	**Family**	**Genus**	**Species**	**Area**	**Australia**	**% Match**	**Assay**	**ESP**	**MAI**	**NIN**	**DAR**	**KI**	**NSI**	**PH**	**YON**	**ROT**
		Liagore		Y		99% (v)	Cr, 16S	0	0	0	0	0	0	0	1	0
		Nanocassiope		M		97%	C3	1	0	0	0	0	0	0	0	7
			Nanocassiope alcocki	N	Y	99-100% (v)	Cr	1	0	0	0	0	0	0	0	3
		Olenothus	*Olenothus uogi*	N	N	99% (v)	Cr	0	0	1	0	0	0	0	0	0
		Paraxanthias	*Paraxanthias elegans*	N	Y	100% (v)	Cr, 16S	0	0	1	0	0	0	0	0	0
		Pilodius	*Pilodius melanospinis*	N	N	98% (v)	Cr, 16S	0	0	3	0	0	2	0	0	0
Euphausiacea				Y		90%	Cr, C3	0	3	0	0	0	2	5	1	0
	Euphausiidae			Y		93-94%	Cr, C3	0	0	0	0	0	3	0	0	6
		Euphausia		M		95-96%	Cn, C3	0	3	1	0	0	0	0	0	0
			Euphausia lucens	Y		98-100% (v)	Cr, M, C3	0	3	0	0	0	0	0	0	0
			Euphausia mutica	Y		99-100% (v)	C3	0	0	0	0	0	4	0	0	0
			Euphausia recurva	Y		98-100% (v)	Cr, 16S, C3	0	1	0	0	0	2	4	0	5
			Euphausia similis	M		100%	Cr, M, C3	0	2	0	0	0	4	0	0	0
		Nyctiphanes		Y		96-97%	M	0	5	0	0	0	0	1	0	0
			Nyctiphanes australis	M		99-100% (v)	Cr, M, 16S, C3	0	9	1	0	5	0	4	0	0
		Thysanoessa	*Thysanoessa gregaria*	S		100% (v)	C3	0	4	0	0	0	2	5	0	0
Isopoda				Y		89%	C3	0	0	0	0	0	0	0	4	0
Mysida				Y		91%	C3	0	0	0	0	0	1	0	5	0
Stomatopoda				Y		91%	M	0	0	1	0	0	0	0	2	0
	Odontodactylidae	*Odontodactylus*	*Odontodactylus latirostris*	N	Y	98%	Cr, 16S	0	0	1	0	0	0	0	0	0
	Protosquillidae	*Chorisquilla*		Y		97%	Cr	0	0	1	0	0	0	0	0	0
		Haptosquilla		Y		95-97%	M, Cr	0	0	3	1	0	0	0	0	0
	Squillidae			Y		92-95%	Cr, 16S	0	0	1	1	0	0	0	5	0
		Oratosquilla		Y		95-97%	Cr, 16S	0	0	0	0	0	0	0	2	0

Sites: ESP – Esperance, MAI – Maria Island, NIN – Ningaloo, DAR – Darwin, KI – Kangaroo Island, NSI – North Stradbroke Island, PH – Port Hacking, YON – Yongala, ROT – Rottnest Island

Assays: 16S – 16S Universal, C3 – Copepod 3, Cn – Cnidaria, Cr – Crustacea, M – Mollusca

Area/Australia: Y – Yes, N – No, M – Mostly, S – Some

(v) Reference sequence taken from a vouchered specimen

Table S4.5: The number of Chordata detections within the Australian zooplankton samples—match and location determined using GenBank (Benson et al. 2014) and ALA (2016) respectively.

Class Order	**Family**	**Genus**	**Species**	**Area**	**Australia**	**% Match**	**Assay**	**ESP**	**MAI**	**NIN**	**DAR**	**KI**	**NSI**	**PH**	**YON**	**ROT**
Chordata (Phyla)				Y		85%	F	0	0	0	2	0	0	1	0	0
Actinopterygii				Y		86-100%	F, M, 16S, Cn, C3	1	0	11	4	1	8	9	13	4
Anguilliformes	Congridae			Y		92%	C3	0	0	0	0	0	0	1	0	0
Aulopiformes	Synodontidae			Y		93-94%	F, 16S	0	0	0	0	0	3	1	2	0
Beloniformes	Exocoetidae			Y		93%	C3	0	0	0	0	1	0	0	0	0
	Scomberesocidae			Y		99-100% (v)	F, C3	0	0	0	0	0	1	1	0	0
		Scomberesox	*Scomberesox saurus saurus*	Y		99-100%	16S	0	0	0	0	0	1	0	0	0
Beryciformes	Holocentridae			Y		92%	M	0	0	0	0	0	0	1	0	0
	Trachichthyidae			Y		93%	F	1	0	0	0	0	1	0	0	1
Clupeiformes				Y		88-91%	F, Cn, C3	0	0	0	5	0	0	0	0	0
	Clupeidae	*Amblygaster*		Y		96-97%	C3	0	0	0	0	0	0	0	1	0
		Sardinella	*Sardinella fimbriata*	N	N	99% (v)	F	0	0	0	1	0	0	0	0	0
		Sardinops		M		96-100%	F	3	2	1	1	7	9	5	6	15
			Sardinops sagax	Y		98-100% (v)	F, M, 16S, Cn, C3	3	3	0	0	8	5	2	3	8
	Dussumieriidae			N	N	94%	F	0	0	0	1	0	0	0	0	0
		Etrumeus	*Etrumeus teres*	Y		98-100%	M, Cn, C3, F. 16S	0	0	0	0	0	4	1	3	7
	Engraulidae	*Engraulis*		Y		98-100% (v)	M, C3, 16S	2	0	0	0	3	4	2	0	2
Characiformes				N	N	91%	C3	0	0	2	0	0	0	0	0	0
Cypriniformes	Cyprinidae			Y		90%	M	0	0	1	0	0	0	0	0	0
Elopiformes	Elopidae	*Elops*		Y		100% (v)	C3	0	0	0	0	0	1	0	0	0
Gadiformes	Merlucciidae	*Macruronus*		Y		100% (v)	M, C3	0	2	0	0	0	0	0	0	0
	Moridae			Y		92-93%	F, C3	1	1	0	0	1	0	2	1	1
		Lotella	*Lotella rhacina*	Y		100% (v)	F, C3	0	2	0	0	0	0	1	0	1
Gasterosteiformes	Pegasidae	*Pegasus*	*Pegasus volitans*	Y		99-100% (v)	F, C3	0	0	0	1	0	0	0	0	0
Lophiiformes	Ceratiidae			Y		93%	C3	0	0	0	0	0	1	2	0	0
Myctophiformes	Myctophidae			Y		94%	16S	0	0	0	0	1	0	0	0	0

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		Ceratoscopelus		Y		97-99%	16S, C3	0	0	1	0	1	0	0	0	0
			Ceratoscopelus warmingii	Y		99-100% (v)	C3	0	0	1	0	0	0	0	0	0
		Diaphus		Y		95-97%	F, 16S	0	0	0	0	0	3	0	0	3
			Diaphus danae	Y		100% (v)	C3	0	0	0	0	0	1	0	0	0
		Gymnoscopelus	*Gymnoscopelus nicholsi*	Y		99%	16S	0	2	0	0	0	0	0	0	0
		Hygophum		Y		97%	F	0	0	0	0	0	0	1	0	0
		Lampadena	*Lampadena speculigera*	S		99-100% (v)	F	0	0	0	0	0	0	1	0	1
		Lampanyctus		Y		97-98%	16S	0	0	0	0	0	0	0	0	1
		Notoscopelus		Y		97%	C3	0	0	0	0	0	1	0	0	0
			Notoscopelus caudispinosus	Y		99%	16S	0	0	0	0	0	1	0	0	0
		Protomyctophum		Y		95-96%	F	0	1	0	0	0	0	0	0	0
		Scopelopsis	*Scopelopsis multipunctatus*	Y		99-100% (v)	16S, C3	0	0	0	0	0	1	1	0	0
Perciformes				Y		89-97%	F, 16S, Cn, C3	2	0	7	3	0	4	5	2	4
	Acanthuridae	*Acanthurus*	*Acanthurus nigrofuscus*	Y		99-100%(v)	F	0	0	1	0	0	0	1	0	0
		Naso		Y		99%	F	0	0	1	0	0	0	0	0	0
	Apogonidae			Y		93%	F	0	0	1	0	0	0	0	0	0
		Apogon		Y		95%	F	0	0	0	0	0	0	0	1	0
			Apogon doederleini	Y		98%	C3	0	0	0	0	0	0	0	0	1
		Ostorhinchus		Y		100% (v)	C3	0	0	1	0	0	0	0	0	0
	Arripidae	*Arripis*		Y		100% (v)	F	0	0	0	0	0	0	1	1	0
	Blenniidae			Y		93%	F	0	0	2	0	0	0	0	0	0
		Cirripectes		Y		96%	F	0	0	0	0	0	0	1	0	1
	Caesionidae	*Caesio*	*Caesio caerulaurea*	Y		100%	F	0	0	1	0	0	1	1	0	0
		Pterocaesio		Y		100% (v)	F	0	0	2	0	0	1	2	0	0
	Callionymidae	*Repomucenus*	*Repomucenus calcaratus*	Y		99-100%	F, 16S, C3	1	0	0	0	0	2	0	0	0
	Carangidae			Y		92-100%	F	0	0	2	0	0	1	0	0	0
		Alepes		Y		100% (v)	C3	0	0	2	0	0	0	0	0	0
			Alepes kleinii	Y		100%	F	0	0	0	1	0	0	0	0	0
		Pseudocaranx		Y		100%	F, 16S	1	0	0	0	0	0	3	1	0
			Pseudocaranx dentex	Y		100% (v)	C3	0	0	0	0	0	0	3	0	0

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			Pseudocaranx wrighti	Y		99%	F	1	0	0	0	0	0	0	0	1
		Trachurus		Y		99%	F, 16S	1	1	0	0	1	4	7	1	1
	Chaetodontidae	*Chaetodon*	*Chaetodon ornatissimus*	Y		100% (v)	F, 16S	0	0	1	0	0	0	0	0	0
	Cheilodactylidae	*Nemadactylus*	*Nemadactylus douglasii*	M		100%	F, 16S	1	0	0	0	0	0	4	0	0
	Chiasmondontidae	*Chiasmodon*		Y		95%	F, 16S	0	0	0	0	0	1	0	0	0
	Clinidae	*Heteroclinus*	*Heteroclinus roseus*	Y		100%	C3	1	0	0	0	0	0	0	0	0
	Drepaneidae	*Drepane*		Y		100%	F	0	0	0	1	0	0	0	0	0
	Eleotridae			Y		93%	C3	0	0	0	2	0	0	0	0	0
		Butis		Y		96%	F	0	0	0	1	0	0	0	0	0
		Hypseleotris	*Hypseleotris compressa*	Y		100% (v)	F	0	0	0	1	0	0	1	0	0
	Emmelichthyidae	*Emmelichthys*	*Emmelichthys nitidus nitidus*	N	N	100% (v)	F	0	2	0	0	0	0	0	0	0
		Plagiogeneion	*Plagiogeneion macrolepis*	Y		100%(v)	C3	0	0	0	0	0	0	0	0	1
			Plagiogeneion rubiginosum	M		99% (v)	F, 16S	0	0	0	0	0	0	0	1	1
	Gasterosteiformes	*Pegasus*	*Pegasus volitans*	Y		99-100% (v)	F, C3	0	0	0	1	0	0	0	0	0
	Gempylidae	*Thyrsites*	*Thyrsites atun*	Y		99-100% (v)	F, 16S	0	0	0	0	2	0	0	0	0
	Gerreidae	*Parequula*	*Parequula melbournensis*	Y		100%	F	1	0	0	0	0	0	0	0	1
	Gobiidae			Y		92-96%	F, 16S, C3	0	0	3	6	0	6	1	4	0
		Amblyeleotris	*Amblyeleotris gymnocephala*	Y		98% (v)	F	0	0	0	0	0	0	0	1	0
		Amblygobius	*Amblygobius phalaena*	Y		99% (v)	F, 16S	0	0	1	0	0	0	0	0	0
		Asterropteryx		Y		97%	F	0	0	1	0	0	0	1	0	0
		Eviota		Y		100% (v)	C3	0	0	2	0	0	0	0	0	0
		Gnatholepis		Y		100%	M	0	0	1	0	0	0	0	0	0
		Gobiodon	*Gobiodon rivulatus*	Y		98-99% (v)	F	0	0	1	0	0	0	0	0	0
		Hazeus		Y		98%	C3	0	0	2	0	0	2	0	3	0
		Valenciennea	*Valenciennea longipinnis*	Y		100% (v)	16S	0	0	1	0	0	0	0	0	0
			Valenciennea puellaris	Y		98-100% (v)	M, C3	0	0	1	0	0	0	0	0	0
	Haemulidae	*Pomadasys*		Y		99-100%	C3	0	0	0	2	0	0	0	0	0
			Pomadasys kaakan	Y		100%	F	0	0	0	2	0	0	0	0	0
	Kyphosidae	*Atypichthys*	*Atypichthys strigatus*	Y		100% (v)	F, 16S, C3	0	0	0	0	0	0	1	0	0

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		Girella	*Girella tricuspidata*	Y		100% (v)	F, C3	0	0	0	0	0	0	2	0	0
		Kyphosus		Y		99-100%	F	0	0	0	0	0	0	0	0	2
		Scorpis		Y		100%	F, 16S	0	0	0	0	0	0	2	0	0
	Labridae			Y		93-97%	F, 16S, C3	2	0	0	0	1	3	4	0	4
		Choerodon	*Choerodon venustus*	Y		99-100%	F, 16S	0	0	0	0	0	3	0	1	0
		Cirrhilabrus		N	Y	100% (v)	C3	0	0	0	0	0	1	0	0	0
		Coris		Y		97%	16S	0	0	0	0	0	0	0	0	1
			Coris auricularis	Y		100% (v)	C3	0	0	0	0	0	0	0	0	1
		Halichoeres	*Halichoeres hartzfeldii*	Y		99%	F, 16S	0	0	0	0	0	1	0	0	0
		Leptojulis	*Leptojulis cyanopleura*	Y		100%	F, 16S	0	0	0	0	0	2	0	0	0
		Notolabrus	*Notolabrus parilus*	Y		99% (v)	F, 16S	0	0	0	0	0	0	1	0	1
		Ophthalmolepis	*Ophthalmolepis lineolata*	Y		100%	F	1	0	0	0	0	0	1	0	0
		Oxycheilinus		Y		97%	F	0	0	1	0	0	0	0	0	0
			Oxycheilinus bimaculatus	Y		99-100% (v)	M, C3	0	0	1	0	0	0	0	0	0
		Pictilabrus	*Pictilabrus laticlavius*	Y		100%	F, 16S	1	1	0	0	0	0	1	0	0
	Labrisomidae			N	N	92%	C3	1	0	0	0	0	0	0	0	3
	Leiognathidae			Y		91-96%	F, M	0	0	0	4	0	1	1	0	0
		Nuchequula	*Nuchequula gerreoides*	Y		98-100% (v)	F	0	0	0	4	0	0	1	0	0
		Secutor	*Secutor megalolepis*	Y		98% (v)	F	0	0	0	4	0	0	0	0	0
	Lethrinidae	*Lethrinus*	*Lethrinus atkinsoni*	Y		99%	F	0	0	1	0	0	0	0	0	0
			Lethrinus laticaudis	Y		100%	F	0	0	0	0	0	0	0	1	0
	Lutjanidae	*Lutjanus*		Y		96-100%	F, C3	0	0	0	2	0	0	0	1	0
	Mullidae			Y		92%	M	0	0	0	0	0	0	0	0	3
		Mulloidichthys		Y		95%	C3	0	0	0	0	0	1	0	0	0
		Upeneichthys	*Upeneichthys stotti*	Y		100%	F	0	0	0	0	0	0	0	0	3
		Upeneus		Y		95-100% (v)	F, Cn, C3	0	0	0	0	0	2	1	7	0
			Upeneus tragula	Y		99-100%	F	0	0	0	0	0	1	0	1	0
	Nemipteridae	*Nemipterus*	*Nemipterus furcosus*	Y		98-100%	C3	0	0	0	0	0	0	0	3	0
			Nemipterus peronii	Y		98-100% (v)	C3	0	0	0	0	0	0	0	1	0
		Scaevius	*Scaevius milii*	Y		98%	M	0	0	1	0	0	0	0	0	0
	Odacidae			Y		93%	F	2	0	0	0	0	0	1	0	0
	Pempheridae	*Parapriacanthus*		Y		95-96%	F, 16S	0	0	0	0	0	1	1	0	0

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			Parapriacanthus elongatus	Y		100%	F	0	0	0	0	0	0	1	1	3
		Pempheris		Y		95-96%	F	0	0	1	0	0	0	0	0	0
	Pinguipedidae	*Parapercis*		Y		100%	16S	1	0	0	0	0	0	0	0	3
			Parapercis haackei	M		100%	F	2	0	0	0	0	0	2	1	3
	Pomacentridae	*Abudefduf*		Y		99% (v)	F	0	0	1	0	0	0	0	0	0
		Chromis		Y		95-96%	F, 16S	0	0	0	0	0	1	0	1	1
			Chromis fumea	N	Y	98%	F	0	0	0	0	0	1	1	0	0
			Chromis notata	N	N	98% (v)	F, C3	0	0	0	0	0	0	1	0	2
		Plectroglyphidodon	*Plectroglyphidodon leucozonus*	Y		100% (v)	M	0	0	1	0	0	0	0	0	0
		Pomacentrus		Y		98-100% (v)	F	0	0	1	0	0	0	0	0	0
			Pomacentrus coelestis	Y		100%	C3	0	0	1	0	0	0	0	0	0
	Scaridae	*Leptoscarus*	*Leptoscarus vaigiensis*	Y		100%	F	0	0	1	0	0	0	0	0	0
		Scarus	*Scarus psittacus*	Y		100%	F	0	0	1	0	0	0	0	1	0
	Schindleriidae	*Schindleria*		Y		95-96%	F, 16S	0	0	2	0	0	0	0	0	0
	Sciaenidae			Y		93-94%	F, C3	0	0	0	3	0	0	0	0	0
	Scombridae			Y		99-100%	F	0	2	0	0	0	0	0	0	0
		Euthynnus		Y		100% (v)	F	0	0	1	0	0	0	1	1	0
		Rastrelliger		Y		99-100% (v)	F	0	0	1	0	0	0	1	0	0
		Scomber		Y		99%	F	0	0	0	0	1	0	0	0	0
			Scomber australasicus	Y		100% (v)	C3	0	0	0	0	2	0	2	0	0
	Serranidae			Y		94%	F, 16S	0	0	0	0	0	2	0	0	0
		Acanthistius	*Acanthistius ocellatus*	Y		99-100% (v)	F, 16S, C3	0	0	0	0	0	0	2	0	2
		Caesioperca	*Caesioperca lepidoptera*	Y		100%	F, 16S	1	3	0	0	0	0	1	0	0
		Cephalopholis		Y		99% (v)	F	0	0	0	1	0	0	0	0	0
		Pseudanthias	*Pseudanthias rubrizonatus*	Y		100%	C3	0	0	0	0	0	2	0	0	0
	Sillaginidae	*Sillago*		Y		96-100%	16S, F, C3	0	0	0	0	0	4	1	1	0
			Sillago bassensis	Y		99-100% (v)	F	1	0	0	0	0	0	0	0	0
			Sillago flindersi	Y		100%	16S	0	0	0	0	0	0	3	0	0
	Sparidae			Y		99%	F	0	0	0	0	0	1	0	0	0
	Terapontidae			Y		92%	C3	0	0	0	1	0	0	0	0	0
		Pelates	*Pelates quadrilineatus*	Y		98%	F, 16S	0	0	0	1	0	0	0	0	0

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	Trichonotidae			Y		94%	C3	0	0	0	0	0	1	0	0	0
Pleuronectiformes	Bothidae	*Lophonectes*	*Lophonectes gallus*	Y		99%	F	0	1	0	0	0	0	0	0	0
	Paralichthyidae	*Pseudorhombus*	*Pseudorhombus dupliciocellatus*	Y		100%	F	0	0	0	0	0	0	0	2	0
Salmoniformes				Y		89%	M	0	0	2	0	0	0	0	0	0
Scorpaeniformes				Y		88-91%	F	2	0	1	0	0	0	1	0	0
	Platycephalidae	*Onigocia*	*Onigocia sibogae*	Y		100%	F	0	0	0	0	0	0	0	1	0
		Platycephalus	*Platycephalus bassensis*	Y		100%	F	0	1	0	0	0	0	0	0	0
			Platycephalus grandispinis	M		99-100%	F	1	0	0	0	0	1	3	1	1
			Platycephalus longispinis	Y		99-100% (v)	C3	0	0	0	0	0	1	1	0	0
			Platycephalus richardsoni	Y		99-100% (v)	F, C3	0	1	0	0	0	0	0	0	0
		Sorsogona	*Sorsogona tuberculata*	Y		100%	F	0	0	0	0	0	0	0	2	0
	Sebastidae			Y		94%	C3	0	0	0	0	0	3	0	0	0
		Helicolenus		Y		98%	F	0	0	0	0	0	0	1	0	0
	Triglidae	*Lepidotrigla*		Y		95-98%	F, 16S	1	1	0	0	0	1	0	0	0
			Lepidotrigla argus	Y		100%	F, 16S, C3	0	0	0	0	0	5	3	0	0
Stomiiformes	Stomiidae	*Rhadinesthes*		N	Y	95%	F, 16S	0	0	0	0	0	1	0	0	0
	Phosichthyidae	*Vinciguerria*		Y		96%	C3	0	0	1	0	0	1	1	0	1
Syngnathiformes	Centriscidae	*Macroramphosus*		Y		100% (v)	C3	0	0	0	0	0	2	0	0	0
			Macroramphosus scolopax	Y		99-100%	F, 16S	0	0	0	0	0	1	0	0	0
Tetraodontiformes	Balistidae			Y		94%	C3	0	0	0	0	0	1	0	0	0
	Monacanthidae			Y		98-99%	F, 16S	1	0	0	0	0	0	0	0	1
		Cantherhines		Y		98-100% (v)	C3	0	0	1	0	0	0	0	0	0
		Paramonacanthus		Y		96%	F, 16S	0	0	0	0	0	0	0	1	0
			Paramonacanthus choirocephalus	Y		98%	F	0	0	0	0	0	0	0	2	0
Aves Charadriiformes	Laridae	*Larus*		Y		99-100% (v)	C3	0	0	0	0	0	0	2	0	0
Leptocardii				N	N	87%	M	3	0	0	0	1	0	0	0	4
Mammalia Carnivora	Canidae	*Canis*	*Canis lupus familiaris*	Y		100%	M, Cn, 16S	0	2	0	0	0	0	1	1	0

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Cetartiodactyla	Balaenopteridae	*Megaptera*	*Megaptera novaeangliae*	Y		98-100% (v)	16S, C3	0	0	2	0	0	1	1	0	2
Primates	Hominidae	*Homo*	*Homo sapiens*	Y		100%	M, 16S, C3	1	3	3	4	2	5	2	1	0
Reptilia Squamata	Gekkonidae	*Hemidactylus*	*Hemidactylus frenatus*	Y		100% (v)	Cn	0	0	0	1	1	0	0	0	0
Thaliacea Doliolida	Doliolidae	*Doliolum*		Y		97%	C3	1	1	0	0	1	3	2	0	1

Sites: ESP – Esperance, MAI – Maria Island, NIN – Ningaloo, DAR – Darwin, KI – Kangaroo Island, NSI – North Stradbroke Island, PH – Port Hacking, YON – Yongala, ROT – Rottnest Island

Assays: 16S – 16S Universal, C3 – Copepod 3, Cn – Cnidaria, F – Fish, M – Mollusca:

Area/Australia: Y – Yes, N – No, M – Mostly, S – Some

(v) Reference sequence taken from a vouchered specimen

Table S4.6: The number of Mollusca detections within the Australian zooplankton samples—match and location determined using GenBank (Benson et al. 2014) and ALA (2016) respectively.

Class Order	**Family**	**Genus**	**Species**	**Area**	**Australia**	**% Match**	**Assay**	**ESP**	**MAI**	**NIN**	**DAR**	**KI**	**NSI**	**PH**	**YON**	**ROT**
Mollusca (phyla)				Y		85-86%	M, Cn, C3	1	0	0	4	3	1	1	11	0
Bivalvia				Y		86-88%	M, 16S, Cn	1	0	3	6	0	13	2	20	2
	Galeommatidae	*Ephippodontina*	*Ephippodontina gigas*	N	N	100% (v)	Cn	0	0	2	0	0	0	0	0	0
Cardiida	Donacidae	Galatea		N	N	96-98%	16S	0	0	0	0	0	0	0	1	0
Ostreida				Y		89-91%	M, Cn	0	0	2	10	0	0	0	2	0
	Ostreidae	*Booneostrea*		Y		95%	M	0	0	3	5	0	0	0	0	0
		Dendostrea	*Dendostrea frons*	N	N	99%	Cn	0	0	0	5	0	0	0	0	0
	Pinnidae			Y		92%	M	0	0	1	4	0	0	0	2	0
		Atrina	*Atrina strangei*	N	Y	100% (v)	M	0	0	0	0	0	2	0	0	0
Cephalopoda Oegopsida	Enoploteuthidae	*Arbralia*		Y		95%	M	0	0	0	0	0	2	0	0	0
		Enoploteuthis	*Enoploteuthis galaxias*	Y		99%	16S	0	0	0	0	0	2	0	0	0
Gastropoda				Y		86-97%	M, 16S, Cn, C3	16	2	24	22	9	14	10	23	16
	Lepetidae			N	Y	92%	C3	0	0	0	0	0	0	0	1	0
	Maningrididae	*Maningrida*	*Maningrida arnhemensis*	Y		98%	Cn	0	0	0	2	0	0	0	0	0
	Plakobranchidae	*Elysia*		Y		95%	M	0	0	1	0	0	0	0	0	0
Aplysiida				Y		90%	M	0	0	0	0	0	0	2	0	0
	Aplysiidae			Y		92%	M	0	1	0	0	3	0	0	0	0
		Aplysia		Y		99% (v)	M	0	0	0	0	0	0	4	0	0
Caenogastropoda				Y		91%	16S	0	0	0	0	1	0	0	0	0
	Cerithiidae	*Bittium*	*Bittium glareosum*	Y		100%	C3	0	0	0	0	0	0	0	7	0
		Cacozeliana	*Cacozeliana granarium*	Y		100%	M	5	1	0	0	1	0	0	0	8
	Epitoniidae			Y		93%	16S	1	0	0	0	0	0	0	0	0
		Epitonium		N	Y	96%	16S	2	0	0	0	0	0	0	0	0
			Epitonium replicatum	S		99-100%	M, 16S	0	0	1	1	0	0	0	1	0
		Janthina		Y		98-100% (v)	16S	0	0	1	0	0	0	0	0	0
			Janthina exigua	Y		100% (v)	16S	0	0	0	0	0	0	1	0	0

Class Order	**Family**	**Genus**	**Species**	**Area**	**Australia**	**% Match**	**Assay**	**ESP**	**MAI**	**NIN**	**DAR**	**KI**	**NSI**	**PH**	**YON**	**ROT**
	Litiopidae	*Litiopa*		Y		97%	C3	0	0	1	0	0	0	1	1	0
	Newtoniellidae	*Ataxocerithium*		Y		97%	C3	1	0	0	0	4	0	0	0	4
	Turritellidae	*Maoricolpus*	*Maoricolpus roseus*	Y		98-100%	C3, M	0	8	0	0	0	0	0	0	0
Cephalaspidea				Y		88-91%	M	0	0	4	1	1	0	0	0	0
	Bullidae	*Bulla*	*Bulla quoyii*	Y		98-100%	M	2	0	0	0	0	0	0	0	0
Cycloneritida	Neritidae	*Neripteron*	*Neripteron violaceum*	N	Y	98%	C3	0	0	0	2	0	0	0	0	0
	Phenacolepadidae			Y		92%	C3	0	0	0	0	0	1	0	0	0
Lepetellida	Haliotidae	*Haliotis*	*Haliotis rubra*	Y		98-100%	C3	1	0	0	0	1	0	0	0	0
Littorinimorpha				Y		89-91%	M, C3	0	1	1	3	1	3	1	1	0
	Atlantidae	*Atlanta*		Y		97%	C3	0	0	0	0	0	1	3	0	0
	Assimineidae			Y		94%	C3	0	0	0	10	0	0	0	0	0
		Cryptassiminea	*Cryptassiminea insolata*	Y		98%	C3	0	0	0	2	0	0	0	0	0
	Calopiidae	*Calopia*	*Calopia minutissima*	Y		100% (v)	M, Cn	0	0	0	9	0	0	0	0	0
	Cypraeidae	*Erosaria*	*Erosaria helvola*	Y		98%	M	0	0	2	0	0	0	0	0	0
	Iravadiidae			Y		95-100%	16S, Cr	0	0	0	2	0	0	0	0	0
		Iravadia		Y		96-100%	M, 16S, Cn	0	0	0	14	0	0	0	0	0
	Littorinidae	*Littoraria*	*Littoraria luteola*	Y		100%	C3	0	0	0	0	0	1	0	0	0
	Naticidae			Y		92%	M	0	0	1	1	0	0	0	0	0
	Pterotracheidae	*Firoloida*		M		97%	C3	0	0	3	0	1	3	6	0	0
	Rissoidae	*Haurakia*	*Haurakia novarensis*	Y		98% (v)	16S	0	0	0	2	0	0	0	0	0
	Strombidae			Y		94%	C3	0	0	0	6	0	2	0	0	0
		Canarium	*Canarium mutabile*	Y		99%	Cn	0	0	2	0	0	0	0	0	0
Neogastropoda				Y		89-91%	M, C3	2	0	8	6	2	2	0	5	3
	Columbellidae	*Amphissa*		N	N	96%	C3	0	0	0	0	0	0	0	2	0
	Conidae	*Conus*	*Conus capitaneus*	Y		98-99% (v)	M	0	0	1	0	0	0	0	0	0
	Costellariidae			Y		94%	C3	0	0	0	5	0	0	0	1	0
	Fasciolariidae			Y		93%	C3	3	0	0	0	3	0	0	0	4
	Muricidae			Y		92%	C3	0	0	0	3	0	0	0	0	0
	Nassariidae			Y		93%	C3	0	0	0	0	0	1	0	0	0
		Nassarius	*Nassarius niger*	N		98% (v)	C3	0	0	0	7	0	0	0	0	0
			Nassarius siquijorensis	N		99-100%	C3	0	0	0	4	0	0	0	0	0
	Terebridae	*Strioterebrum*		Y		97%	C3	0	0	0	0	0	0	0	2	0

Class Order	**Family**	**Genus**	**Species**	**Area**	**Australia**	**% Match**	**Assay**	**ESP**	**MAI**	**NIN**	**DAR**	**KI**	**NSI**	**PH**	**YON**	**ROT**
	Turridae			Y		92-93%	M, C3	0	0	2	3	0	1	0	0	1
Nudibranchia				Y		88-91%	M, Cn	2	0	2	1	2	2	1	4	2
	Chromodorididae			Y		92%	M	0	0	2	0	0	1	0	0	0
	Polyceridae			Y		93%	M	0	0	0	4	0	0	0	0	0
		Kaloplocamus		N	Y	95%	M	0	0	0	1	0	0	0	0	0
Pleurobranchomorpha	Pleurobranchidae	*Pleurobranchus*		Y		100% (v)	Cn	0	0	1	0	0	0	0	0	0
Pteropoda	Creseidae			Y		94%	M	8	1	2	0	7	9	12	0	6
		Creseis		M		98%	M	2	0	4	10	0	3	3	11	3
	Limacinidae	*Heliconoides*	*Heliconoides inflatus*	Y		99% (v)	M, Cn	1	4	0	0	1	0	2	0	1
Trochida	Turbinidae	*Lunella*	*Lunella torquata*	Y		99-100%	C3	0	0	0	0	1	0	0	0	0
Umbraculida	Umbraculidae	*Umbraculum*	*Umbraculum umbraculum*	Y		98%	M	0	0	0	0	1	0	0	0	0
Polyplacophoda Chitonida	Mopaliidae			Y		92%	C3	0	0	1	0	0	0	0	0	0

Sites: ESP – Esperance, MAI – Maria Island, NIN – Ningaloo, DAR – Darwin, KI – Kangaroo Island, NSI – North Stradbroke Island, PH – Port Hacking, YON – Yongala, ROT – Rottnest Island

Assays: 16S – 16S Universal, C3 – Copepod 3, Cn – Cnidaria, Cr – Crustacea, M – Mollusca

Area/Australia: Y – Yes, N – No, M – Mostly, S – Some

(v) Reference sequence taken from a vouchered specimen

Table S4.7: The number of Cnidaria detections within the Australian zooplankton—match and location determined using GenBank (Benson et al. 2014) and ALA (2016) respectively.

Class Order	**Family**	**Genus**	**Species**	**Area**	**Australia**	**% Match**	**Assay**	**ESP**	**MAI**	**NIN**	**DAR**	**KI**	**NSI**	**PH**	**YON**	**ROT**
Cnidaria (phyla)				Y		85%	M	0	0	0	0	1	0	0	0	0
Anthozoa Actiniaria				Y		93-100%	M, Cn, C3	4	0	0	6	0	2	0	0	2
Alcyonacea				Y		99-100%	Cn	0	0	0	1	0	0	1	0	0
	Ellisellidae			Y		99-100% (v)	Cn	0	0	0	0	0	0	0	1	0
	Nephtheidae			Y		100%	Cn	0	0	1	0	0	1	1	2	0
Scleractinia				Y		100% (v)	M	0	0	1	1	0	0	0	0	0
	Caryophylliidae	*Caryophyllia*	*Caryophyllia (Caryophyllia) diomedeae*	Y		99%	C3	1	0	0	0	0	0	2	0	0
Pennatulcea	Virgulariidae	*Virgularia*	*Virgularia schultzei*	N	N	100%	Cn	0	0	0	0	0	0	0	1	0
Zoantharia				Y		99-100%	M	3	0	0	1	2	0	0	0	0
	Zoanthidae			Y		98%	M	0	0	1	4	0	0	0	0	0
Hydrozoa				Y		86-94%	M, Cn, C3	5	5	7	10	4	4	1	8	7
Anthoathecata				Y		89-90%	Cn	4	0	0	0	3	0	0	0	5
	Pandeidae			N	Y	92-93%	Cn	0	0	0	2	1	0	0	1	0
		Amphinema		N	Y	95-97%	M, Cn	0	0	0	7	0	0	0	0	0
	Rathkeidae	*Lizzia*	*Lizzia blondina*	N	N	100%	M	0	3	0	0	3	0	5	0	0
Leptothecata				Y		88-94%	M, Cn	0	0	7	11	0	0	0	7	1
	Bonneviellidae			N	N	92%	Cn	0	0	0	1	0	0	0	0	0
	Campanulariidae	*Clytia*		M		97-98% (v)	M	2	4	0	0	3	0	0	0	4
		Obelia		Y		94-95%	M	0	3	0	0	0	0	0	0	0
	Laodiceidae	*Laodicea*		N	N	94-95%	Cn	0	0	1	0	0	0	0	3	0
	Lovenellidae			N	Y	92-93%	Cn	0	0	0	2	0	0	0	0	0
Siphonophorae				Y		90-91%	Cn	0	2	3	0	0	0	4	0	1
	Agalmatidae			Y		96%	C3	0	0	0	9	0	0	0	0	0
		Athorybia	*Athorybia rosacea*	N	N	99%	Cn	0	0	1	0	0	0	0	0	0
		Nanomia		S		97%	Cn	0	0	3	0	0	8	4	2	3
			Nanomia bijuga	N	N	99%	C3	0	0	2	0	0	2	5	0	1

Class Order	**Family**	**Genus**	**Species**	**Area**	**Australia**	**% Match**	**Assay**	**ESP**	**MAI**	**NIN**	**DAR**	**KI**	**NSI**	**PH**	**YON**	**ROT**
	Abylidae	*Bassia*		N	N	97%	Cn	0	0	2	0	0	4	3	0	0
			Bassia bassensis	N	N	100%	Cn	1	0	2	0	0	4	4	0	3
	Diphyidae	*Lensia*	*Lensia subtiloides*	N	N	100%	M	2	0	6	1	0	2	2	6	4
	Sphaeronectidae	*Sphaeronectes*	*Sphaeronectes koellikeri*	N	N	100%	M, Cn	3	9	6	1	6	11	9	6	7
Trachymedusae	Geryoniidae	*Liriope*	*Liriope tetraphylla*	M		100%	C3	3	0	0	5	0	4	0	8	10
	Rhopalonematidae	*Aglaura*	*Aglaura hemistoma*	M		100%	Cn	1	0	3	0	3	6	5	0	4

Sites: ESP – Esperance, MAI – Maria Island, NIN – Ningaloo, DAR – Darwin, KI – Kangaroo Island, NSI – North Stradbroke Island, PH – Port Hacking, YON – Yongala, ROT – Rottnest Island

Assays: C3 – Copepod 3, Cn – Cnidaria, M – Mollusca

Area/Australia: Y – Yes, N – No, M – Mostly, S – Some

(v) Reference sequence taken from a vouchered specimen

Table S4.8: The number of Echinodermata detections within the Australian zooplankton samples—match and location determined using GenBank (Benson et al. 2014) and ALA (2016) respectively.

Class Order	**Family**	**Genus**	**Species**	**Area**	**% Match**	**Assay**	**ESP**	**MAI**	**NIN**	**DAR**	**KI**	**NSI**	**PH**	**YON**	**ROT**
Echinodermata (phyla)				Y	84-86%	Cn, C3	5	0	0	0	0	9	3	0	6
Asteroidea				Y	87%	Cn, C3	0	0	0	0	0	0	0	5	1
Valvatida				Y	90-91%	Cn, C3	1	0	0	0	0	4	1	0	1
Echinoidea				Y	86-89%	M, Cn	3	0	1	3	0	8	1	5	7
Camarodonta	Echinometridae	*Heliocidaris*	*Heliocidaris erythrogramma*	Y	100% (v)	Cn	0	0	0	0	0	0	1	0	0
	Temnopleuridae	*Salmaciella*	*Salmaciella oligopora*	Y	100%	Cn	0	0	0	0	0	3	0	3	0
		Salmacis	*Salmacis belli*	Y	100%	Cn	0	0	0	2	0	0	0	1	0
			Salmacis sphaeroides	Y	100%	C3	0	0	0	0	0	0	0	3	0
		Temnopleurus	*Temnopleurus michaelseni*	Y	99-100%	M, Cn, C3	5	0	0	0	1	0	0	0	8
Cidaroida	Cidaridae	*Prionocidaris*		Y	97%	Cn	0	0	0	0	0	0	0	0	1
Clypeasteroida				Y	88-89%	Cn	0	0	1	0	0	0	0	0	0
	Clypeasteridae	*Clypeaster*	*Clypeaster japonicus*	Y	98%	Cn	0	0	0	0	0	1	0	0	0
Diadematoida				Y	89%	16S	0	0	0	0	0	0	0	0	1
Spatangoida	Loveniidae			Y	92-94%	M, Cn	4	1	0	0	0	0	0	0	0
		Echinocardium		Y	97%	Cn	0	4	0	0	0	2	1	0	0
			Echinocardium cordatum	Y	100%	M	0	6	0	0	0	2	3	0	0
Holothuroidea Apodida				Y	89%	C3	1	0	0	0	0	0	0	0	0
Dendrochirotida				Y	89-90%	M	1	1	0	0	0	0	0	0	0
Synallactida				Y	89%	Cn	0	0	0	3	0	8	2	0	0
Ophiuroidea				Y	87-88%	Cn, C3	2	2	1	2	0	0	5	0	4
Amphilepidida	Ophiactidae	*Ophiactis*	*Ophiactis resiliens*	Y	99%	M	1	0	0	0	1	0	5	0	0
	Ophionereididae			Y	92%	C3	0	0	0	4	0	0	0	0	0
		Ophionereis	*Ophionereis schayeri*	Y	99-100%	Cn	1	0	0	0	0	0	0	0	1
	Ophiotrichidae	*Ophiothrix*		Y	95%	Cn	0	0	0	0	0	0	2	0	0
			Ophiothrix (Ophiothrix) aristulata	Y	99-100%	C3	0	1	0	0	0	0	0	0	0

Class Order	**Family**	**Genus**	**Species**	**Area**	**% Match**	**Assay**	**ESP**	**MAI**	**NIN**	**DAR**	**KI**	**NSI**	**PH**	**YON**	**ROT**
			Ophiothrix (Ophiothrix) caespitosa	Y	100%	M, Cn	0	3	0	0	5	0	2	0	0
Ophiurida				Y	88-91%	Cn, C3	0	0	0	2	0	8	0	12	2
	Ophiuridae	*Ophiura*	*Ophiura kinbergi*	Y	100%	Cn	2	2	0	0	4	1	0	0	0

Sites: ESP – Esperance, MAI – Maria Island, NIN – Ningaloo, DAR – Darwin, KI – Kangaroo Island, NSI – North Stradbroke Island, PH – Port Hacking, YON – Yongala, ROT – Rottnest Island
Assays: 16S – 16S Universal, C3 – Copepod 3, Cn – Cnidaria, M – Mollusca;
Area/Australia: Y – Yes
(v) Reference sequence taken from a vouchered specimen

Table S4.9: The number of Animalia detections (not noted elsewhere) within the Australian zooplankton samples—match and location determined using GenBank (Benson et al. 2014) and ALA (2016) respectively.

Class Order	**Family**	**Genus**	**Species**	**Area**	**Australia**	**% Match**	**Assay**	**ESP**	**MAI**	**NIN**	**DAR**	**KI**	**NSI**	**PH**	**YON**	**ROT**
Animalia (kingdom)				Y		88-93%	Cn, C3	6	0	2	12	9	8	0	4	11
Annelida (phyla)				Y		87-91%	M, C3	1	0	3	0	0	0	0	0	0
Polychaeta				Y		86-88%	M, Cn	0	0	1	7	3	0	1	0	1
	Chaetopteridae	*Chaetopterus*		Y		100% (v)	M	0	0	0	0	0	5	2	0	1
	Sabellariidae	*Idanthyrsus*	*Idanthyrsus australiensis*	Y		100%	M	0	0	0	0	0	0	2	0	0
Phyllodocida				Y		89-90%	M	0	0	0	2	0	0	0	0	0
Sabellida	Serpulidae	*Vinearia*		Y		96%	C3	0	0	0	0	0	1	0	0	0
Bryzoa (phyla)	**Gymnolaemata** (class)			Y		91%	C3	0	0	2	0	0	0	0	0	0
Cheilostomatida	Membraniporidae	*Biflustra*		Y		97%	M	0	0	2	6	0	0	0	2	1
		Membranipora	*Membranipora membranacea*	S		99-100% (v)	Cr, M, C3	2	5	0	0	8	0	1	0	0
Ctenostomatida				N	N	91%	C3	0	0	2	0	0	0	0	0	0
	Flustrellidridae	*Flustrellidra*		N	N	96%	C3	0	0	2	10	0	0	1	7	0
			Flustrellidra armata	N	N	99%	M	0	0	3	8	0	2	3	7	0
Chaetognatha (phyla)				Y		85%	C3	0	0	0	1	0	0	0	0	0
Sagittiodea				M		86-88%	C3	0	0	3	14	0	0	0	0	1
Aphragmophora				Y		89-90%	M, Cn, C3	0	0	2	16	0	7	1	3	0
	Sagittidae			M		92-94%	M, C3	1	0	4	2	0	9	2	11	2
		Flaccisagitta		M		95-97%	C3	5	0	8	4	0	3	1	11	16
			Flaccisagitta enflata	M		98-100%	C3	11	0	17	12	0	24	8	33	32
		Sagitta		M		95-97%	M, Cn, C3	2	0	4	10	0	4	1	5	7

Class Order	**Family**	**Genus**	**Species**	**Area**	**Australia**	**% Match**	**Assay**	**ESP**	**MAI**	**NIN**	**DAR**	**KI**	**NSI**	**PH**	**YON**	**ROT**
Hemichordata (phyla)	**Enteropneusta** (class)			M		86-88%	M, Cn, C3	0	0	1	3	0	0	0	0	0
Nemertea (phyla)				Y		86-94%	C3	1	0	0	5	0	0	1	1	0
Anopla				N	Y	89-90%	Cn, M	2	0	0	1	0	0	1	0	0
	Lineidae			N	Y	93%	C3	0	0	0	0	0	0	0	0	1
Palaeonemertea				N		87%	Cn	0	0	0	2	0	0	0	0	0
	Carinomidae			Y		92%	C3	0	0	0	0	0	0	0	1	0
	Cephalothricidae			M		92-93%	M	0	0	0	0	0	2	0	1	0
Enopla				Y		88%	M	0	0	0	0	0	1	0	1	0
Monostilifera				N	N	90-91%	C3	0	0	0	2	0	0	0	0	0
	Amphiporidae	*Poseidonemertes*		N	Y	95%	C3	0	0	0	0	0	0	1	0	1
	Plectonemertidae			Y		94%	C3	0	0	0	0	0	0	0	0	1
	Tetrastemmatidae	*Tetrastemma*		N	Y	95%	C3	0	0	0	0	0	1	0	0	0
Platyhelminthes (phyla)				Y		87-88%	M	0	0	0	1	0	0	1	0	0
Rhabditophora Polycladida	Stylochoplanidae			N	N	92%	Cn	0	0	0	1	0	0	0	0	0
Porifera (phyla)																
Demospongiae Clionaida	Clionaidae	*Cliona*	*Cliona jullieni*	N	N	98%	M	1	0	0	0	1	0	0	0	1
Dendroceratida	Darwinellidae	*Dendrilla*		Y		99-100% (v)	C3	0	0	0	0	0	0	0	0	1
Poecilosclerida	Dendoricellidae	*Fibulia*	*Fibulia cribriporosa*	N	N	98-99%	M	0	1	0	0	0	0	0	0	0

Sites: ESP – Esperance, MAI – Maria Island, NIN – Ningaloo, DAR – Darwin, KI – Kangaroo Island, NSI – North Stradbroke Island, PH – Port Hacking, YON – Yongala, ROT – Rottnest Island
Assays: C3 – Copepod 3, Cn – Cnidaria, M – Mollusca
Area/Australia: Y – Yes, N – No, M – Mostly, S - Some
(v) Reference sequence taken from a vouchered specimen

Table S4.10: The number of Eukaryota detections (not noted elsewhere) within the Australian zooplankton samples—match and location determined using GenBank (Benson et al. 2014) and ALA (2016) respectively.

Phyla/Class	**Class**/Order	Family	Genus /Species	Area	Australia	% Match	Assay	ESP	MAI	NIN	DAR	KI	NSI	PH	YON	ROT
Chromista (kingdom)																
Haptophyta	**Prymnesiophyceae**			Y		86%	M	0	1	0	0	0	0	0	0	0
Myzozoa	**Dinophyceae**			S		94-100% (v)	Cn	2	8	0	2	11	1	2	2	2
Ochrophyta				Y		85%	C3	0	0	0	1	0	0	0	0	0
Bacillariophyceae	Thalassiosirales			Y		90-91%	Cn	0	0	0	0	0	3	8	0	0
Dictyochophyceae				Y		89%	Cn	0	2	0	0	0	0	0	0	0
Raphidophyceae	Chattonellales	Chattonellaceae	*Heterosigma akashiwo*	N	N	99-100%	Cn	0	1	0	0	0	0	0	0	0
Oomycota	**Peronosporea**			Y		89%	Cn	1	4	0	0	0	2	8	0	0
Fungi (kingdom)																
Ascomycota	**Dothideomycetes**			Y		95-96%	Cn	0	0	0	0	1	0	0	1	1
Plantae (kingdom)																
Chlorophyta				Y		85%	M	0	0	1	0	0	5	1	0	0
Chlorophyceae	Chlamydomonadales			Y		89%	M	0	0	0	0	0	0	1	0	0
Mamiellophyceae				N	N	88%	M	0	0	1	0	0	7	6	0	1
	Mamiellales	Bathycoccaceae	*Bathycoccus prasinos*	N	N	99%	M	0	1	1	0	0	0	0	0	0
Prasinophyceae				N	Y	87-100%	M, Cn	22	13	28	14	11	60	43	33	50
Pyramimonadophyceae	Prasinococcales	Prasinococcaceae	*Prasinoderma coloniale*	N	N	99%	Cn	5	1	1	0	1	10	7	1	5
Rhodophyta	**Bangiophyceae**			M		100%	C3	1	1	1	0	1	2	3	0	0
Florideophyceae	Ceramiales			Y		91-92%	Cn	0	0	0	0	0	0	0	0	1

Sites: ESP – Esperance, MAI – Maria Island, NIN – Ningaloo, DAR – Darwin, KI – Kangaroo Island, NSI – North Stradbroke Island, PH – Port Hacking, YON – Yongala, ROT – Rottnest Island
Assays: C3 – Copepod 3, Cn – Cnidaria, M – Mollusca
Area/Australia: Y – Yes, N – No, M – Mostly, S – Some
(v) Reference sequence taken from a vouchered specimen

Table S4.11: The taxa identified as the most characteristic—present in the majority of the samples from one site but mostly absent in the others—of each site (*Indval*; Roberts, 2016; $p < 0.01$)

Site	Taxa	Indicator Value	P value	Site	Taxa	Indicator Value	P value	Site	Taxa	Indicator Value	p value
Ningaloo	*Metalpheus paragracilis*	0.5714	0.001	Darwin	*Parvocalanus crassirostris*	1.0000	0.001	Yongala	*Bittium glareosum*	0.5833	0.001
Ningaloo	*Thalamita admete*	0.5291	0.001	Darwin	*Iravadia*	0.9167	0.001	Yongala	Macrophthalmus	0.5104	0.001
Ningaloo	Haptosquilla	0.3588	0.003	Darwin	Assimineidae	0.8333	0.001	Yongala	Mollusca	0.4255	0.001
Ningaloo	Annelida	0.3086	0.002	Darwin	Agalmatidae	0.7500	0.001	Yongala	*Gebiacantha plantae*	0.4167	0.002
Ningaloo	Kochlorine	0.3086	0.003	Darwin	*Calopia minutissima*	0.7500	0.001	Yongala	Mysida	0.3472	0.002
Ningaloo	Galatheidae	0.3086	0.004	Darwin	Eukaryota	0.6722	0.001	Yongala	Asteroidea	0.3472	0.002
Ningaloo	*Pilodius melanospinis*	0.3086	0.004	Darwin	*Amphinema*	0.5833	0.001	Yongala	*Candacia discaudata*	0.3387	0.001
Ningaloo	*Lensia subtiloides*	0.2970	0.004	Darwin	*Nassarius niger*	0.5833	0.001	Yongala	Isopoda	0.3333	0.003
Ningaloo	Cephalaspidea	0.2884	0.009	Darwin	Sagittoidea	0.5162	0.001	Yongala	Bivalvia	0.3196	0.001
Ningaloo	Ctenostomatida	0.2857	0.010	Darwin	Penaeidae	0.5104	0.001	Yongala	Ophiurida	0.2807	0.003
Ningaloo	Blenniidae	0.2857	0.010	Darwin	Ostreida	0.4678	0.001	Yongala	*Acartia*	0.2701	0.009
Ningaloo	*Alepes*	0.2857	0.008	Darwin	Tortanidae	0.4167	0.001	Yongala	*Labidocera acuta*	0.2584	0.001
Ningaloo	*Eviota*	0.2857	0.009	Darwin	*Dendostrea frons*	0.4167	0.001	Yongala	*Upeneus*	0.2541	0.009
Ningaloo	*Schindleria*	0.2857	0.010	Darwin	Aphragmophora	0.3934	0.001	Yongala	*Creseis*	0.2432	0.004
Ningaloo	Salmoniformes	0.2857	0.008	Darwin	Flustrellidra	0.3853	0.001	Yongala	*Subeucalanus*	0.2234	0.009
Ningaloo	*Canarium mutabile*	0.2857	0.009	Darwin	Strombidae	0.3750	0.001	Yongala	*Centropages furcatus*	0.2207	0.001
Ningaloo	*Candacia truncata*	0.2818	0.002	Darwin	Costellariidae	0.3472	0.001	Yongala	*Canthocalanus pauper*	0.2186	0.001
Ningaloo	*Farranula gibbula*	0.2818	0.001	Darwin	Scyllaridae	0.3333	0.004	Maria Is	*Maoricolpus roseus*	0.7273	0.001
Ningaloo	Oncaeidae	0.2761	0.003	Darwin	Clupeiformes	0.3333	0.003	Maria Is	*Calocalanus tenuis*	0.6206	0.001
Ningaloo	Perciformes	0.2748	0.003	Darwin	*Secutor megalolepis*	0.3333	0.004	Maria Is	*Calanus*	0.5319	0.001
Ningaloo	*Calocalanus styliremis*	0.2698	0.006	Darwin	Ophionereididae	0.3333	0.002	Maria Is	*Evadne nordmanni*	0.4842	0.001
Ningaloo	*Calocalanus minutus*	0.2697	0.003	Darwin	*Nassarius siquijorensis*	0.3333	0.004	Maria Is	*Calanus australis*	0.4236	0.001
Ningaloo	Neogastropoda	0.2649	0.006	Darwin	Polyceridae	0.3333	0.001	Maria Is	*Neocalanus tonsus*	0.3929	0.001
Ningaloo	*Clausocalanus minor*	0.2190	0.002	Darwin	Polychaeta	0.2649	0.008	Maria Is	*Clausocalanus ingens*	0.3891	0.001
Ningaloo	Paracalanidae	0.2051	0.009	Darwin	Ocypodidae	0.2604	0.007	Maria Is	*Nyctiphanes*	0.3726	0.001
Ningaloo	*Acartia negligens*	0.1887	0.010	Darwin	*Metapenaeopsis*	0.2604	0.009	Maria Is	*Nyctiphanes australis*	0.3371	0.001
Ningaloo	*Paracalanus aculeatus*	0.1840	0.003	Darwin	Leptothecata	0.2440	0.009	Maria Is	Temoridae	0.3157	0.004
Nth Stradbroke Is	*Portunus sanguinolentus*	0.4167	0.001	Darwin	*Biflustra*	0.2414	0.008	Maria Is	*Echinocardium cordatum*	0.2940	0.003
Nth Stradbroke Is	Synallactida	0.3980	0.001	Port Hacking	*Haloptilus longicornis*	0.6095	0.001	Maria Is	*Dardanus arrosor*	0.2852	0.003
Nth Stradbroke Is	Euphausia_mutica	0.3333	0.003	Port Hacking	*Aplysia*	0.4000	0.001	Maria Is	*Neocalanus*	0.2727	0.002
Nth Stradbroke Is	Calanidae	0.3191	0.001	Port Hacking	*Clausocalanus jobei*	0.3402	0.001	Maria Is	Metridinidae	0.2727	0.004

Site	Taxa	Indicator Value	P value
Nth Stradbroke Is	*Copilia mirabilis*	0.2893	0.008
Nth Stradbroke Is	*Subeucalanus pileatus*	0.2545	0.002
Nth Stradbroke Is	Cyclopoida	0.2512	0.003
Nth Stradbroke Is	*Centropages orsinii*	0.2178	0.006
Kangaroo Is	*Membranipora membranacea*	0.5297	0.001
Kangaroo Is	*Ophiothrix caespitosa*	0.3558	0.002
Kangaroo Is	*Sardinops sagax*	0.3025	0.001
Kangaroo Is	Aplysiidae	0.3018	0.006
Kangaroo Is	*Ataxocerithium*	0.2500	0.008
Kangaroo Is	*Sardinops*	0.2079	0.009
Rottnest Is	*Nanocassiope*	0.4537	0.001
Rottnest Is	Euphausiidae	0.3333	0.003
Rottnest Is	*Subeucalanus subtenuis*	0.3150	0.001
Rottnest Is	Trapeziidae	0.2976	0.007
Rottnest Is	*Etrumeus teres*	0.2686	0.001
Rottnest Is	*Liriope tetraphylla*	0.2525	0.001

Site	Taxa	Indicator Value	P value
Port Hacking	*Pseudevadne tergestina*	0.3225	0.003
Port Hacking	*Clausocalanus pergens*	0.3173	0.001
Port Hacking	Podonidae	0.3158	0.002
Port Hacking	*Ophiactis resiliens*	0.3158	0.004
Port Hacking	*Trachurus*	0.3096	0.001
Port Hacking	*Pseudocaranx dentex*	0.3000	0.006
Port Hacking	*Sillago flindersi*	0.3000	0.001
Port Hacking	*Scolecithrix danae*	0.2976	0.001
Port Hacking	*Pleuromamma abdominalis*	0.2946	0.003
Port Hacking	*Nemadactylus douglasii*	0.2824	0.009
Port Hacking	*Lucicutia flavicornis*	0.2661	0.003
Port Hacking	*Temora discaudata*	0.2655	0.002
Port Hacking	*Firoloida*	0.2565	0.007
Port Hacking	Lucicutiidae	0.2524	0.004
Port Hacking	Euphausiacea	0.2444	0.008
Port Hacking	*Thysanoessa gregaria*	0.2426	0.008
Port Hacking	*Nanomia bijuga*	0.2414	0.005
Port Hacking	*Atlanta*	0.2348	0.008
Port Hacking	*Paracalanus tropicus*	0.2099	0.005

Site	Taxa	Indicator Value	p value
Maria Is	*Euphausia lucens*	0.2727	0.003
Maria Is	*Obelia*	0.2727	0.003
Maria Is	*Podon intermedius*	0.2489	0.007
Esperance	Loveniidae	0.5867	0.001
Esperance	*Temnopleurus michaelseni*	0.4274	0.001
Esperance	*Cacozeliana granarium*	0.4047	0.001
Esperance	*Crangon*	0.3333	0.005
Esperance	*Bulla quoyii*	0.3333	0.002
Esperance	*Pseudevadne*	0.3158	0.002
Esperance	Anthoathecata	0.3048	0.004
Esperance	Echinodermata	0.3019	0.001
Esperance	Zoantharia	0.3000	0.004
Esperance	Actiniaria	0.2963	0.005
Esperance	Leptocardii	0.2609	0.007
Esperance	Odacidae	0.2564	0.008
Esperance	*Penilia avirostris*	0.2554	0.005

Table S4.12: The significance of differences found between the assemblage (composition: below the diagonal) and richness (number: above the diagonal) of the operational taxonomic units (OTUs) between the sampling sites—PERMANOVA (Anderson et al., 2008)

Site	Assay	Ningaloo	Darwin	Yongala	North Stradbroke Island	Port Hacking	Maria Island	Kangaroo Island	Esperance	Rottnest Island
Ningaloo	16S Uni		-	-	-	-	-	-	*	-
	Cnidaria		-	**	-	-	**^{HB}	*	-	-
	Copepod 3		**	*	-	-	**	***^{HB}	*	-
	Crustacea		-	-	-	*	***^{HB}	**	**^{HB}	-
	Fish		*	-	-	-	**	**	-	-
	Mollusca		-	***^{HB}	-	-	***^{HB}	***^{HB}	*	**
Darwin	16S Uni	***^{HB}		-	*	-	-	-	*	-
	Cnidaria	***^{HB}		-	**	*	*	-	-	-
	Copepod 3	***^{HB}		-	***^{HB}	***^{HB}	-	***^{HB}	-	**
	Crustacea	***^{HB}		-	-	-	*	-	-	-
	Fish	**		*	**	***^{HB}	-	*	-	*
	Mollusca	***^{HB}		**^{HB}	-	-	***^{HB}	***^{HB}	*	*
Yongala	16S Uni	***^{HB}	***^{HB}		*	-	-	-	*	-
	Cnidaria	***^{HB}	***^{HB}		***^{HB}	***^{HB}	-	-	-	-
	Copepod 3	***^{HB}	***^{HB}		***^{HB}	**^{HB}	-	**^{HB}	-	*
	Crustacea	***^{HB}	***^{HB}		-	-	***^{HB}	*	*	-
	Fish	**	***^{HB}		-	-	*	**	-	-
	Mollusca	***^{HB}	***^{HB}		**	**	*	-	-	-
Nth Stradbroke Island	16S Uni	***^{HB}	***^{HB}	***^{HB}		**	**	**	**	**
	Cnidaria	***^{HB}	***^{HB}	***^{HB}		-	***^{HB}	***^{HB}	-	*
	Copepod 3	***^{HB}	***^{HB}	***^{HB}		-	**^{HB}	***^{HB}	**^{HB}	-
	Crustacea	***^{HB}	***^{HB}	***^{HB}		*	***^{HB}	**	**^{HB}	-
	Fish	**^{HB}	***^{HB}	**		-	**^{HB}	***^{HB}	-	-
	Mollusca	***^{HB}	***^{HB}	***^{HB}		-	***^{HB}	**	-	-
Port Hacking	16S Uni	***^{HB}	***^{HB}	***^{HB}	***^{HB}	Richness → Assemblage ←	-	-	-	-
	Cnidaria	***^{HB}	***^{HB}	***^{HB}	***^{HB}		***^{HB}	***^{HB}	-	-
	Copepod 3	***^{HB}	***^{HB}	***^{HB}	***^{HB}		**^{HB}	***^{HB}	**^{HB}	-
	Crustacea	***^{HB}	***^{HB}	***^{HB}	***^{HB}		-	-	-	-
	Fish	**^{HB}	***^{HB}	***^{HB}	**		**^{HB}	***^{HB}	-	*
	Mollusca	***^{HB}	***^{HB}	***^{HB}	***^{HB}		***^{HB}	**	-	*
Maria Island	16S Uni	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}		-	-	-
	Cnidaria	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}		-	-	*
	Copepod 3	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}		-	-	**
	Crustacea	***^{HB}	***^{HB}	***^{HB}	***^{HB}	**^{HB}		*	-	***^{HB}
	Fish	**	**^{HB}	***^{HB}	**^{HB}	**^{HB}		-	-	**
	Mollusca	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}		-	**	*
Kangaroo Island	16S Uni	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}		-	-
	Cnidaria	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}		-	-
	Copepod 3	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}		*	***^{HB}
	Crustacea	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}		-	*
	Fish	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	**		*	***^{HB}
	Mollusca	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}		-	-
Esperance	16S Uni	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	*		*
	Cnidaria	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}		-
	Copepod 3	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}		*
	Crustacea	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	**^{HB}		*
	Fish	*	**	*	*	-	-	-		-
	Mollusca	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}		-
Rottnest Island	16S Uni	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	
	Cnidaria	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	-	
	Copepod 3	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	
	Crustacea	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	
	Fish	***^{HB}	***^{HB}	**^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	-	
	Mollusca	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	***^{HB}	

*** ($p \leq 0.001$) ** ($p \leq 0.01$) * ($p \leq 0.05$) and ^{HB} Holm-Bonferroni correction

Table S4.13: Results of a cross validation test—where a sample is removed and replaced using the assemblage of the OTUs within the sample—between the sampling sites—CAP (Anderson et al., 2008)

Classified Site ➔	Esperance	Maria Is	Ningaloo	Darwin	Kangaroo Is	Nth Stradbroke Is	Port Hacking	Yongala	Rottnest Is	% Correct
Originating Site ↓	16S Universal									
Esperance	1	-	-	-	3	-	-	-	2	17
Maria Is	-	10	-	-	-	-	1	-	-	91
Ningaloo	-	-	6		-	1	-	-	-	86
Darwin	-	-	-	12	-	-	-	-	-	100
Kangaroo Is	2	-	-	-	6	-	-	-	-	75
Nth Stradbroke Is	-	-	-	-	-	12	-	-	-	100
Port Hacking	-	-	-	-	2	1	7	-	-	70
Yongala	-	-	-	-	-	2	-	10	-	83
Rottnest Is	1	-	-	-	-	-	-	-	11	92
Total correct		75/90								83.33
Originating Site ↓	Cnidaria									
Esperance	3	-	-	-	-	-	-	-	3	50
Maria Is	-	11	-	-	-	-	-	-	-	100
Ningaloo	-	-	7	-	-	-	-	-	-	100
Darwin	-	-	-	11	-	-	-	-	-	100
Kangaroo Is	-	2	-	-	6	-	-	-	-	75
Nth Stradbroke Is	-	-	-	-	-	12	-	-	-	100
Port Hacking	-	-	-	-	-	3	7	-	-	70
Yongala	-	-	-	-	-	-	-	12	-	100
Rottnest Is	6	-	-	-	-	-	-	-	6	50
Total correct		75/89								84.17
Originating Site ↓	Copepod 3									
Esperance	4	-	-	-	-	-	-	-	2	67
Maria Is	-	9	1	-	-	-	-	-	-	90
Ningaloo	-	-	5	1	-	1	-	-	-	71
Darwin	-	-	-	10	-	-	-	-	-	100
Kangaroo Is	-	-	-	-	7	-	-	-	-	100
Nth Stradbroke Is	-	-	-	-	-	12	-	-	-	100
Port Hacking	-	-	-	-	-	1	9	-	-	90
Yongala	-	-	-	-	-	-	-	12	-	100
Rottnest Is	3	-	-	-	-	-	-	-	9	75
Total correct		77/86								89.54

Classified Site ➔	Esperance	Maria Is	Ningaloo	Darwin	Kangaroo Is	Nth Stradbroke Is	Port Hacking	Yongala	Rottnest Is	% Correct
Originating Site ↓					Crustacea					
Esperance	6	-	-	-	-	-	-	-	-	100
Maria Is	-	7	-	-	2	-	2	-	-	64
Ningaloo	-	-	6	-	-	1	-	-	-	86
Darwin	-	-	-	10	-	-	1	1	-	83
Kangaroo Is	1	-	-	-	7	-	-	-	-	88
Nth Stradbroke Is	-	-	-	-	-	10	1	-	1	83
Port Hacking	-	-	-	-	1	2	7	-	-	70
Yongala	-	1	-	-	-	1	-	10	-	83
Rottnest Is	2	-	-	-	-	-	-	1	9	75
Total correct		72/90								80
Originating Site ↓					Fish					
Esperance	1	1	-	-	2	-	1	-	1	17
Maria Is	1	4	-	-		-	-	2	-	57
Ningaloo	-	-	5	-	1	-	-	-	-	83
Darwin	-	-	-	9	1	-	-	2	-	75
Kangaroo Is	1	-	-	-	6	-	-	1	-	75
Nth Stradbroke Is	-	-	-	-	1	3	3	3	2	25
Port Hacking	1	-	1	1	-	2	4	-	1	40
Yongala	-	-	2	-	-	1	-	6	2	55
Rottnest Is	-	-	-	-	2	1	-	-	9	75
Total correct		47/84								55.95
Originating Site ↓					Mollusca					
Esperance	3	-	-	-	-	-	-	-	3	50
Maria Is	-	11	-	-	-	-	-	-	-	100
Ningaloo	-	-	6		-	1	-	-	-	86
Darwin	-	-	-	11	-	-	-	-	-	100
Kangaroo Is	-	-	-	-	8	-	-	-	-	100
Nth Stradbroke Is	-	-	-	-	-	11	1	-	-	92
Port Hacking	-	-	-	-	-	2	8	-	-	80
Yongala	-	-	-	-	-	-	-	12	-	100
Rottnest Is	-	3	-	-	-	-	-	-	8	73
Total Correct		78/88								88.64

Table S4.14: The significance of the seasonal differences found within each site—PERMANOVA (Anderson et al., 2008).

Assay	OTU diversity	Ningaloo	Darwin	Yongala	North Stradbroke Island	Port Hacking	Maria Island	Kangaroo Island	Esperance	Rottnest Island
16S Universal	Richness	-	-	-	-	-	-	*	-	-
	Assemblage	*	-	-	*	-	-	-	-	*
Cnidaria	Richness	-	*	-	*	-	-	-	-	-
	Assemblage	*	-	-	**	*	-	-	-	***
Copepod3	Richness	-	-	-	**	-	-	-	-	-
	Assemblage	-	-	*	**	**	-	-	-	*
Crustacea	Richness	-	-	-	-	-	-	-	-	-
	Assemblage	-	-	-	*	-	-	-	-	-
Fish	Richness	-	-	-	-	-	-	-	-	*
	Assemblage	-	-	-	-	-	-	-	-	-
Mollusca	Richness	-	-	-	*	-	-	-	-	-
	Assemblage	-	*	-	**	**	-	-	-	**

*** ($p \leq 0.001$) ** ($p \leq 0.01$) * ($p \leq 0.05$)

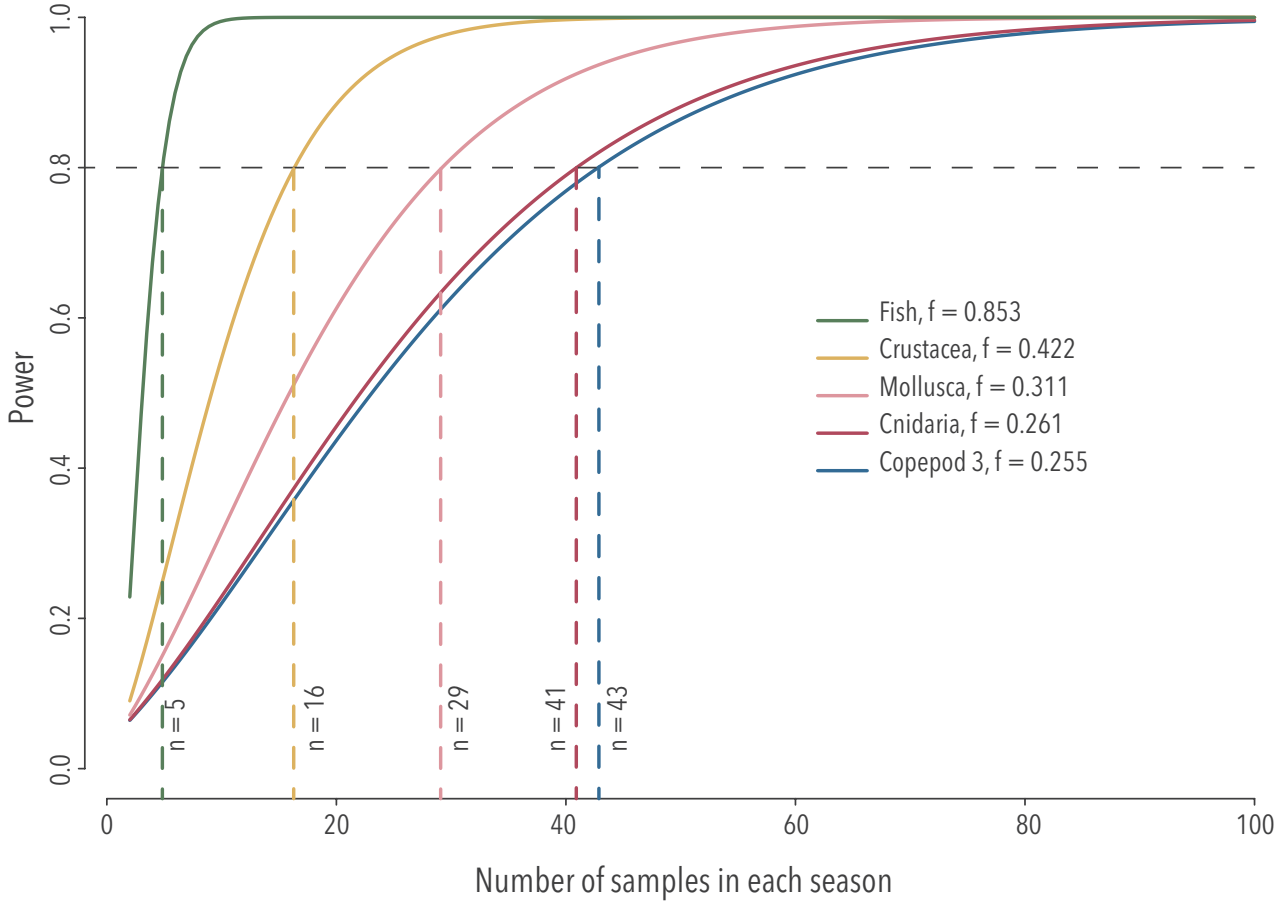

Figure S4.1: Power curves from each assay indicating the number of samples required to detect seasonal changes in Richness. Thirteen samples per season sampled from Rottnest Island were used to create the curves (created in R—R Core Team, 2015, from PERMANOVA Richness data—Anderson et al., 2008). Power levels of 0.8 are indicated with the corresponding number of samples per season required to achieve this.

Table S4.15: The significance of Assemblage differences at Rottnest Island using a varying number of samples per season—PERMANOVA (Anderson et al., 2008)

Assay	Overall Significance	Summer Spring	Summer Winter	Summer Autumn	Spring Winter	Spring Autumn	Winter Autumn
One sample per season over three years – Three samples per season							
Cnidaria	***	-	-	-	-	-	-
Copepod 3	*	-	-	-	-	-	-
Crustacea	-	-	-	-	-	-	-
Fish	-	-	-	-	-	-	-
Mollusca	**	-	-	-	-	-	-
One sample per season over five years – Five samples per season							
Cnidaria	*	*	**	-	-	-	-
Copepod 3	**	-	** HB	-	** HB	-	-
Crustacea	***	** HB	** HB	-	*	-	-
Fish	**	-	** HB	-	*	*	-
Mollusca	***	-	** HB	-	** HB	-	*
Two samples per season over five years – Ten samples per season							
Cnidaria	***	** HB	*** HB	** HB	*	** HB	-
Copepod 3	***	** HB	*** HB	** HB	*** HB	** HB	-
Crustacea	***	** HB	*** HB	** HB	** HB	** HB	* HB
Fish	***	-	*** HB	*** HB	*** HB	*** HB	** HB
Mollusca	***	** HB	*** HB	** HB	*** HB	** HB	** HB
Three samples per season over five years – Twelve samples per season							
Cnidaria	***	* HB	*** HB	** HB	*** HB	*** HB	** HB
Copepod 3	***	*	*** HB	* HB	*** HB	*** HB	-
Crustacea	***	** HB	*** HB	* HB	** HB	*** HB	* HB
Fish	***	** HB	*** HB	** HB	*** HB	*** HB	*** HB
Mollusca	***	* HB	*** HB	** HB	*** HB	*** HB	*** HB
Three samples per season over three years – Nine samples per season							
Cnidaria	***	* HB	*** HB	** HB	** HB	** HB	-
Copepod 3	***	* HB	*** HB	** HB	*** HB	*** HB	** HB
Crustacea	***	-	** HB	-	** HB	*	-
Fish	***	** HB	*** HB	** HB	* HB	** HB	* HB
Mollusca	***	** HB	*** HB	*** HB	** HB	*** HB	** HB

*** ($p \leq 0.001$) ** ($p \leq 0.01$) * ($p \leq 0.05$) and ^{HB}Holm-Bonferroni correction

ALA. 2016. *Atlas of Living Australia website* [Online]. http://www.ala.org.au. [Accessed 2017].

ANDERSON, M., GORLEY, R. N. & CLARKE, R. K. 2008. *Permanova+ for Primer: Guide to Software and Statisticl Methods*, Primer-E Limited.

BENSON, D. A., CLARK, K., KARSCH-MIZRACHI, I., LIPMAN, D. J., OSTELL, J. & SAYERS, E. W. 2014. GenBank. *Nucleic Acids Research,* 42**,** D32-D37.

BERRY, T. E., OSTERRIEDER, S. K., MURRAY, D. C., COGHLAN, M. L., RICHARDSON, A. J., GREALY, A. K., STAT, M., BEJDER, L. & BUNCE, M. 2017. DNA metabarcoding for diet analysis and biodiversity: A case study using the endangered Australian sea lion (*Neophoca cinerea*). *Ecol Evol,* 7**,** 5435-5453.

BERRY, T. E., SAUNDERS, B. J., COGHLAN, M. L., STAT, M., JARMAN, S., RICHARDSON, A. J., DAVIES, C. H., BERRY, O., HARVEY, E. S. & BUNCE, M. 2019. Marine environmental DNA biomonitoring reveals seasonal patterns in biodiversity and identifies ecosystem responses to anomalous climatic events. *PLOS Genetics,* 5(2). e1007943

DEAGLE, B. E., GALES, N. J., EVANS, K., JARMAN, S. N., ROBINSON, S., TREBILCO, R. & HINDELL, M. A. 2007. Studying Seabird Diet through Genetic Analysis of Faeces: A Case Study on Macaroni Penguins (*Eudyptes chrysolophus*). *PLoS ONE,* 2**,** e831.

ROBERTS, D. W. 2016. labdsv: Ordination and Multivariate Analysis for Ecology. R package version 1.8-0.

R CORE TEAM. 2015. *R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria* [Online]. Vienna, Austria. Available: http://www.R-project.org/. [Accessed 2016].

VENABLES, W. & RIPLEY, B. 2002. Random and mixed effects. *Modern applied statistics with S.* Springer.

4.10 Close

THIS Australia wide study of zooplankton community eDNA produced clear and significant spatial patterns with an abundance of interesting taxa. Of particular note were the detections of the Humpback whales (Figure 4.4) and the two invasive species revealed at several of the NRS stations. Their inclusion within the eDNA data demonstrates the potential significance of genetic biomonitoring in the management marine environments, particularly with regard to endangered and/or invasive species.

Relative to the Rottnest Island data presented in Chapter Three, the decision to limit the number of samples at each station resulted in a noticeable loss of temporal resolution at the sites. Unfortunately the expense, in time, funds and resources, prevented the analysis of more samples for this Australia wide study. There are several hundred zooplankton samples remaining in the IMOS collection—these data speak to the value that would result if the funding were available to analyse these genetic time capsules. Ultimately the decision to subsample seasons and years stymied the evaluation, but the resources provided by the extended temporal data from Rottnest Island (Chapter Three) offered the opportunity to contrast those results with the Australia wide data. This comparison provides a guide to the amount of sampling required to see significant patterns in zooplankton community assemblages over time. The inclusion of the simultaneously obtained abiotic data delivered confirmation of their ongoing importance to the spatial and temporal changes seen within the zooplankton communities.

Figure 4.4: A young humpback whale breaching *(Megaptera novaeangliae*). One of the rare finds within the zooplankton eDNA data. (Photo by Kelly Minogue (2017), reprinted with permission)

This chapter provides evidence for the potential of the techniques it embodies. The use of high throughput sequencing and appropriately selected multi-gene metabarcoding assays offers a tangible avenue for global applications in the ongoing biomonitoring of our oceans and their biota. Yet what was discovered in this chapter is but a mere 'drop in the ocean'. Collectively, Chapters Two, Three and Four have shown that broad scale sampling for eDNA can be relatively easy to collect, reveals both temporal and spatial patterns and can identify cryptic and hidden taxa, and as such is destined to be an essential part of any biomonitoring program. Additionally, Chapters Three and Four demonstrated that the integration of concurrent abiotic data could provide the opportunity for future modelling to help inform ecological management decisions.

Chapter Five provides a summation of what was achieved during the tenure of this thesis, and speculates on the significance this research has had, is having and how it is likely to shape future research in this field.

CHAPTER – FIVE

The end of a beginning

If we knew what it was we were doing, it would not be called research, would it?

Albert Einstein

I have my own views about Nature's methods, though I feel that it is rather like a beetle giving his opinions upon the Milky Way.

'Sherlock Holmes'

Sir Arthur Conan Doyle

5.1 In retrospect

THROUGHOUT the tenure of this thesis, the research was confined to the marine environment, yet it could have just as easily focussed on desert, mangrove or another type of ecosystem. The overwhelming advantage of metabarcoding methodologies is that—using the appropriate assays—they can be applied to environmental DNA (eDNA) sourced from virtually any biological environment.

The purpose of this chapter is to review and conclude this thesis, summarising the 'what' and 'why' of the research carried out, and addressing the 'what next' in respect of the multitude of potential future directions available.

5.1.1 Thesis recapitulation & significance

It is well established that enduring spatial and temporal monitoring is necessary to produce information for marine management strategies and to track the ongoing evolution of marine environments (Matz et al., 2018). Yet the difficulty and expense of traditional methods can prevent the very research required to meet this need. Extended spatial and temporal biomonitoring using multi-gene metabarcoding of eDNA is a relatively cost effective way to contrast and compare the effects of changing conditions within and between different areas.

When this research commenced there were very few effective tools to explore marine eDNA. The chance to work with several opportunistic marine samples was provided and, using these, extraction methods were refined and a suite of assays to probe the DNA was developed. This resulted in the production of tens of millions of metabarcode sequences for analysis, which were then translated into thousands of Operational Taxonomic Units (OTUs) and the identification of thousands of taxa. The utility of the molecular 'tool kit' for use with several environmental substrates was

demonstrated, but, ultimately, almost as many questions as were answered, were generated. Nevertheless, the research proved the capacity of these approaches to obtain useful, robust and often otherwise unobtainable data from eDNA. The integration of these molecular methods with more traditional biotic and abiotic data will provide a powerful approach for biodiversity assessment going forward.

In Chapter Two, using scat from the Australian sea lion (*Neophoca cinerea*), a valuable understanding of the dietary habits of this endangered endemic pinniped was formed, as well as an insight into the biodiversities of its environments. This was the first next generation molecular foray into sea lion diet, and it was demonstrated that, given a large enough number of samples, the collection of scat from known sea lion haul out points furnishes sufficient eDNA to enable a comprehensive yet non-invasive dietary survey. New insights into sea lion feeding habits were gleaned and several new prey that were hitherto unknown to science were identified. The applied research also demonstrated the importance of screening each scat sample to confirm the species of origin.

An expansion of this study across several years to include both a wider range of haul out beaches and to collect samples on a monthly or seasonal basis would provide practical and useful knowledge of the dietary preferences of the sea lion and its surrounding biodiversity. This information is particularly important for the Australian sea lion, as each colony habitually returns to the same breeding and haul out points and they are not known to expand their range. The data obtained could be used to gauge how adaptable the species is to a changing diet and therefore aid in the conservation and management of the species, the ecosystems it inhabits and the often-conflicting interests of industry.

A recent example of the need for up-to-date scientific information for management purposes was exposed in an article from an online edition of ABC News (Logan, 2018). Here Western Australian fishers claimed the introduction of gillnet-free zones to protect the Australian sea lion was “unjustified”, but the Australian Fisheries

Management Authority argued the nets were a "major threat to sea lion population recovery". One argument used was that sea lions eat mainly cuttlefish, octopus and squid and not so much the fish and shark that are targeted by the fishers. In contrast, our study (Berry et al., 2017—Chapter Two) found that the Australian sea lion was an opportunistic benthic forager and while the study confirmed that half the sea lion's diet is made up of cephalopods (on a presence/absence basis), the remainder consisted of other taxa, particularly fish and sharks. However our study was not extensive and more data would be required to draw conclusive findings on the sea lion's dietary preferences. The salient point being that metabarcoding methods can bring empirical data to debates such at this, and is highly relevant to the making of informed decisions for management strategies.

The third and fourth chapters both originated from bulk-plankton samples provided by the Integrated Marine Observing System (IMOS). Chapter Three involved an extensive temporal study using 50 samples taken from the Rottnest Island National Reference Station (NRS) across five years. This study demonstrated that, typical (seasonal) and atypical (heatwave) changes in the zooplankton community from this site could be mapped using multi-gene metabarcoding methods. The range of taxa identified was vast and yet there were almost as many sequences that could not be assigned to any useful taxonomic level. Indicator OTUs were identified for each time period providing a genetic characterisation of diversity across each of the time periods examined. In addition, the effects that such a heatwave can have on the assemblage of zooplankton in a relatively short period of time were revealed. This is particularly important in the light of global warming, as changes in plankton assemblages may have cascading effects on the animals that rely on plankton for food.

Chapter Four employed samples from all nine Australian IMOS NRS with the aim of expanding the study from Chapter Three across a wide spatial scale. The sampling incorporated a variety of zooplankton communities taken from the tropical waters near Darwin to the temperate waters near Hobart (Figure 4.1). A total of 90 samples taken seasonally across three years also provided the opportunity to test for temporal patterns

at each site. The results revealed striking spatial patterns, with clear and highly significant delineations of zooplankton community assemblages between all nine sites—producing an average of 80% correct sample replacement in a cross validation test across all assays. However the limited numbers of samples taken from within each site (constrained by budget and time restraints) restricted the detection of significant site-based temporal differences. The subsequent integration of the Rottnest data from Chapter Three provided a valuable guide to the extent of sampling required to observe significant temporal changes within the data. For this study, a site based indicator analysis was performed on the assigned taxa, providing evidence for the key species characterising each NRS.

While the range of taxa detected within both these studies was vast there were many sequences that were unassignable using the current sequence reference databases. It is recognised that282 both primer bias and reference database limitations currently restrict identification of much of the zooplankton communities sampled. Both will improve with time and further research.

5.1.2 Future research opportunities

The methods devised during the study are already in use for other projects. A side study undertaken during the tenure of this thesis involved developing methods for the extraction and analysis of eDNA from seawater. This pilot side study has resulted in several publications including two co-author papers (Simpson et al., 2016, Stat et al., 2017). Further, the methods used in Chapter Two were used in a second author publication (Hardy et al., 2017). Copies of these papers 'as published' (where permitted), or the abstract (where not), are contained in the Appendix to this thesis. These expansions of the work undertaken during this thesis provide added evidence of the value of the methods devised. Nevertheless, there is still more work that could be done in the future.

While the methods developed and used in Chapter Two are proven for use to study the Australian sea lion diet—and could easily be applied to a future expansion of the pilot study, they could also be applied to other marine predators. Another potential opportunity would be the investigation of suitable Single Nucleotide Polymorphisms (SNPs) to allow for the tracking and identification of genetic variants within the sea lion populations. As scat abounds with host DNA (an issue that must be overcome in dietary studies), the collection of scat from known haul out points would provide a non-invasive way to monitor sea lion communities, their genetic diversity and any gene flow between the individual colonies—all vital information for the management of this endangered species. This type of study could be run concurrent with dietary and biodiversity studies using scat as the eDNA substrate.

The suite of assays developed for Chapters Three and Four proved that an abundance of information can be obtained from an appropriately preserved plankton sample. The obvious advancement of these studies is for an ongoing temporal extension of the Australia-wide study. This would allow for the patterns and responses in the zooplankton communities to be mapped and potentially predicted using more advanced modelling incorporating oceanic and climate data. These models in turn could be used to provide evidence for the maintenance and future management of marine ecosystems in response to natural and anthropogenic biotic pressures. Unfortunately, IMOS has currently suspended the sampling of plankton for genetic purposes, leaving a large gap in the ongoing chronology. For the sake of future science, it is my opinion that, this decision must be reversed and I hope the findings within this thesis will validate the importance of ongoing sampling.

One of the more interesting finds in the bulk-plankton (see Chapter Four) was the detection of the Humpback whales (*Megaptera novaeangliae*) in a total of six samples; two samples from Rottnest Island and Ningaloo, and one sample from North Stradbroke Island and Port Hacking. All detections occurred during the whales' annual migration pattern between late autumn and spring—they were not detected outside these times. The discovery of whales was quite unexpected but nevertheless

demonstrated the power of the methodologies to detect a mega-fauna from shed eDNA alone. The finding opens the possibility for the use of plankton samples to source eDNA and SNPs for the genetic monitoring of Humpback whales. A particularly interesting project would be to combine their genetic diversity and migration patterns. A similar study on haplotype diversity in metabarcoding and eDNA data was recently carried out on whale Sharks (Sigsgaard et al., 2016) and lethrinid Fish (Stat et al., 2017; see Appendix).

The research presented in Chapters Three and Four merely skimmed the surface of the data collected. For example, several samples have been extracted and sequenced two or three times: during the pilot study; again for the Rottnest study; and finally for the Australia-wide study. A comparison of these sequences could provide an interesting estimation as to the consistency of the data obtained from a homogenised plankton sample. All samples in this study were amplified in duplicate, but an evaluation of those samples extracted and amplified more than once, could provide an indication of whether this duplication alone is sufficient to extract all the information available to each assay—or whether more extractions are required. As a field that is still developing, there is a clear need to continue to develop metabarcoding methods from sample collection, through to sample processing, library building and filtering to ensure the fidelity of the data. Even over the relatively short tenure of this thesis there have been noticeable changes in how metabarcoding workflows are implemented and which assay(s) are appropriate.

The final table (5.1) is a consolidated review of the 'tool kit' used during this thesis. It provides a brief overview of some of the advantages and disadvantages of single and multi assay approaches, the pros and cons for each assay used and finally the advantages and disadvantages of using a taxonomic or taxonomy free (OTU) approach to analysis.

Table 5.1: Summary of some of the advantages and disadvantages of the eDNA tool kits used in this thesis

Approaches used	Advantages	Disadvantages
Single vs. multi-assay approach		
Single 'universal' assay	Easily processed Cost effective Provides the capability to study large numbers of samples Depending on the assay can be good for limited range of taxa or can give a broad idea of the taxa present	Primer In a mixed eDNA sample, risk of missing important taxa Species identification is unlikely unless the assay is targeting a highly variable barcode
Multi assay approach	Broader range of taxa targeted Can provide a better understanding of the overall biodiversity contained within a sample	More difficult to process and analyse Often a detections overlap between assays Increased cost per sample limits the number of samples used Still not a complete picture of biodiversity
Assays (target sequence length without primers)		
16S Universal (Deagle et al. 2007) ~ 180-270 bp	Detects across a wide range of taxa including Malacostraca, Actinopterygii, Mammalia and some Mollusca Can identify many taxa to species level	Doesn't detect Hexanauplia Inappropriate for mammalian dietary studies Length of barcode limits use with highly degraded DNA
Bird 12S (Cooper, 1994) ~ 230 bp	Specific for Avians Good discriminatory power for species assignment	Only detects birds Limited species recovery from degraded DNA
Ceph 16S (Berry et al. 2017) ~ 200 bp	Targets Cephalopoda Good discriminatory power for species assignment	Limited species recovery from degraded DNA
Cnidaria (Berry et al. 2019) COI ~ 145 bp	Targets Hydrozoa and some Echinoidea	Limited Anthozoa detection
Copepod 1 (Berry et al. 2019) COI ~ 131 bp	Primarily targets Hexanauplia and some mollusca Short enough for degraded DNA	Some redundancy and not as much data when compared with Copepod 3
Copepod 2 (Berry et al. 2019) COI ~ 134 bp	Primarily targets Hexanauplia and some mollusca Short enough for degraded DNA	Some redundancy and not as much data when compared with Copepod 3
Copepod 3 (Berry et al. 2019) COI~ 100bp	Detects an extensive range of taxa to genus and species Not confined to copepods Useful for degraded DNA Gives a broad understanding of the biodiversity present in the sample	Short target sequence can limit discrimination power between related species

Approaches used	Advantages	Disadvantages
Crustacea (Crust 16S) (Berry et al. 2017) ~ 170 bp	Detects a wide range of Malacostraca Able to determine to genera or species level for many taxa	Really only detects Malacostraca
Fish (Deagle et al. 2007 & Berry et al. 2017) 16S ~ 200 bp	Specific for fish Detects a broad range of Actinopterygii Detects some Chondrichthyes Good species detection	Only detects Fish
Mam 16S (Taylor, 1996) ~ 90 bp	Specific for Mammalia Good discrimination power between species	Only detects Mammals
Mollusca (Berry et al. 2019) COI ~ 118 bp	Short barcode Detects a range of Mollusca and other zooplankters	
Plank COI (Berry et al. 2015) ~ 127 bp	Detects a range of Chondrichthyes Detects some Actinopterygii	Limited plankton detection
S_Ceph (Peters et al. 2014) 16S ~ 70 bp	Targets Cephalopoda Useful for degraded DNA	Poor discrimination power between species
Universal (Pochon et al. 2013) 18S ~ 400bp	Broad detection across many phyla	18S is often conserved across related species Reliable taxonomic identification often limited to a family level Less suitable for degraded DNA
OTUs vs. Taxonomic assignment		
Taxonomic assignment	Gives an idea of the range of taxa present in a sample Can hone in on specific taxa	Loss of data, sometimes less than half the unique sequences obtained are identified
OTUs	Uses all the data obtained from a sample Suitable for statistical analysis Can later be taxonomically identified if needed (and able)	Cannot answer questions about specific taxa Potential overlap in data therefore difficult to combine results from multiple assays By grouping taxa by (e.g. 97%) similarity, related species may be missed.

5.1.3 The beginning draws to a close

The results presented in this thesis pave the way for new avenues for discovery. The marine biota is abundant and incredibly diverse, and much of it is, as yet, undescribed. While the studies presented within this thesis have opened but a small window into the marine biosphere, they offer several innovative and exciting methods with which to explore our marine habitats. On December 20 the United Nations (2017) announced a new initiative—'a decade of ocean science'—that is advocating for a renewed and combined focus on ocean research. UNESCO stated:

> *Nearly 3 billion people depend on marine and coastal biodiversity to meet their needs. It absorbs around a third of the CO2 produced by humans and reduces the impact of climate change. However, science has not yet managed to fully evaluate the cumulative effects of human activities on the ocean, including the impact of pollution, warming and acidification, which threaten this environment, which is vital for our survival.*

Clearly, mapping our ocean's biota and predicting how it will change in the near future is vital to marine management. The research in this thesis has attempted to build some methodological approaches in the marine metabarcoding field and, in doing so, has provided data that will help inform best-practice biomonitoring and management. I am privileged to have had the chance to be a part of these developments, and I thank you for reading about my journey.

Everything has to come to an end, sometime.

L. Frank Baum

The Marvelous Land of Oz

BERRY, O., BULMAN, C., BUNCE, M., COGHLAN, M., MURRAY, D. C. & WARD, R. D. 2015. Comparison of morphological and DNA metabarcoding analyses of diets in exploited marine fishes. *Marine Ecology Progress Series,* 540**,** 167-181.

BERRY, T. E., OSTERRIEDER, S. K., MURRAY, D. C., COGHLAN, M. L., RICHARDSON, A. J., GREALY, A. K., STAT, M., BEJDER, L. & BUNCE, M. 2017. DNA metabarcoding for diet analysis and biodiversity: A case study using the endangered Australian sea lion (*Neophoca cinerea*). *Ecol Evol,* 7**,** 5435-5453.

BERRY, T. E., SAUNDERS, B. J., COGHLAN, M. L., STAT, M., JARMAN, S., RICHARDSON, A. J., DAVIES, C. H., BERRY, O., HARVEY, E. S. & BUNCE, M. 2019. Marine environmental DNA biomonitoring reveals seasonal patterns in biodiversity and identifies ecosystem responses to anomalous climatic events. *PLOS Genetics,* 5(2). e1007943

DEAGLE, B. E., GALES, N. J., EVANS, K., JARMAN, S. N., ROBINSON, S., TREBILCO, R. & HINDELL, M. A. 2007. Studying Seabird Diet through Genetic Analysis of Faeces: A Case Study on Macaroni Penguins (*Eudyptes chrysolophus*). *PLoS ONE,* 2**,** e831.

HARDY, N., BERRY, T., KELAHER, B. P., GOLDSWORTHY, S. D., BUNCE, M., COLEMAN, M. A., GILLANDERS, B. M., CONNELL, S. D., BLEWITT, M. & FIGUEIRA, W. 2017. Assessing the trophic ecology of top predators across a recolonisation frontier using DNA metabarcoding of diets. *Marine Ecology Progress Series,* 573**,** 237-254.

COOPER, A. 1994. *DNA from museum specimens,* New York, Springer.

INTERGOVERNMENTAL OCEANOGRAPHIC COMMISION OF UNESCO. 2017. 20 December 2017. *Updates to voluntary commitment.* oceanconference.un.org:.

LOGAN, T. 2018. Gillnet-free zones to begin in WA sea lion recovery areas leading to fears fishers will be forced out. ABC News Rural.

MATZ, M. V., TREML, E. A., AGLYAMOVA, G. V. & BAY, L. K. 2018. Potential and limits for rapid genetic adaptation to warming in a Great Barrier Reef coral. *PLoS Genet,* 14**,** e1007220.

PETERS, K. J., OPHELKELLER, K., HERDINA, BOTT, N. J. & GOLDSWORTHY, S. D. 2015. PCR-based techniques to determine diet of the Australian sea lion (*Neophoca cinerea*): a comparison with morphological analysis. *Marine Ecology,* 36**,** 1428-1439.

SIGSGAARD, E. E., NIELSEN, I. B., BACH, S. S., LORENZEN, E. D., ROBINSON, D. P., KNUDSEN, S. W., PEDERSEN, M. W., JAIDAH, M. A., ORLANDO, L., WILLERSLEV, E., MØLLER, P. R. & THOMSEN, P. F. 2016. Population characteristics of a large whale shark aggregation inferred from seawater environmental DNA. *Nature Ecology & Evolution,* 1**,** 0004.

SIMPSON, T. J., DIAS, P. J., SNOW, M., MUNOZ, J. & BERRY, T. 2016. Real-time PCR detection of *Didemnum perlucidum* (Monniot, 1983) and *Didemnum vexillum* (Kott, 2002) in an applied routine marine biosecurity context. *Mol Ecol Resour*.

STAT, M., HUGGETT, M. J., BERNASCONI, R., DIBATTISTA, J. D., BERRY, T. E., NEWMAN, S. J., HARVEY, E. S. & BUNCE, M. 2017. Ecosystem biomonitoring with eDNA: metabarcoding across the tree of life in a tropical marine environment. *Sci Rep,* 7**,** 12240.

TAYLOR, P. G. 1996. Reproducibility of Ancient DNA Sequences from Extinct Pleistocene Fauna. *Molecular Biology and Evolution,* 13**,** 283-285.

Appendix

A home for lost things

Science is not only a discipline of reason but, also, one of romance and passion

Stephen Hawking

Science is the best idea humans ever had. The more people who embrace that idea, the better

Bill Nye

Every reasonable effort has been made to acknowledge the owners of copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

6.1 Chapter Two—Permissions

DNA metabarcoding for diet analysis and biodiversity: A case study using the endangered Australian sea lion (*Neophoca cinerea*), 2017, *Ecology and Evolution,* Vol. 7(14), pg. 5435-5453

Tina Berry, Sylvia Osterrieder, Dáithí Murray, Megan Coghlan, Anthony Richardson, Alicia Grealy, Michael Stat, Lars Bejder and Michael Bunce.

TB was involved with all aspects of the design and implementation of the experiment and the production of the manuscript. MC provided valuable assistance in the laboratory. LB and MB conceived the idea for the project and SO collected the samples. DM aided with data filtration and designed an assay. AG created the figures. AR instigated the statistical analysis. All authors contributed their expertise to edit and refine the paper but this was particularly so for MB and MS.

I, Sylvia Parsons (nee Osterrieder), confirm my contribution to the cited publication and give permission for its inclusion in this thesis.

Signature | 

Date | 18 July 2018

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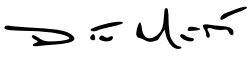
Signature | 

Date | 31 July 2018

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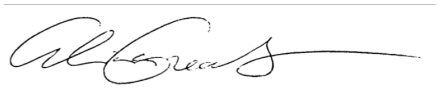
Signature | 

Date | 17 July 2018

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Date | 14 August 2018

6.1 Chapter Three—Permissions

MARINE environmental DNA biomonitoring reveals seasonal patterns in biodiversity and identifies ecosystem responses to anomalous climatic events, 2019, *PLOS Genetics*, 15(2)

Tina E. Berry, Benjamin J Saunders, Megan L. Coghlan, Michael Stat, Simon Jarman, Anthony J. Richardson, Claire H. Davies, Oliver Berry, Euan S. Harvey, and Michael Bunce.

T.E.B., O.B., A.J.R., M.B. and M.S. conceived and designed the study. C.D. and A.J.R. facilitated access to the samples and abiotic data. T.E.B. and M.L.C. refined the approach and produced the data. B.S., T.E.B., E.S.H. and A.J.R designed and produced the statistical analysis. T.E.B., S.J., M.B., M.S. and B.S. discussed the results in preparation of the manuscript. All authors were involved in the final editing of the manuscript.

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Date | 8 August 2018

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Date | 29 July 2018

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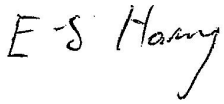
Signature | 

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Date | 14 August 2018

6.1 Chapter Four—Permissions

A three year, Australia-wide, environmental DNA study reveals spatial and temporal patterns in marine biodiversity .

Tina E Berry, Megan L Coghlan, Benjamin J Saunders, Simon Jarman, Matthew Power, Anthony J Richardson, Claire Davies, Oliver Berry and Michael Bunce.

T.E.B., O.B., A.J.R. and M.B. conceived and designed the study. C.D. and A.J.R. facilitated access to the samples and abiotic data. T.E.B., M.L.C and M.P. refined the approach and produced the data. T.E.B., B.S. and A.J.R designed and produced the statistical analysis. T.E.B., S.J., M.B., M.L.C and B.S. discussed the results in preparation of the manuscript. All authors will be involved in the final editing of the manuscript.

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Date | 14 August 2018

ALEXANDER, J. B., BUNCE, M., WHITE, N., WILKINSON, S. P., ADAM, A. S. A., **BERRY, T.**, STAT, M., THOMAS, L., NEWMAN, S. J., DUGAL, L. & RICHARDS, Z. T. (2019) Development of a multi-assay approach for monitoring coral diversity using eDNA metabarcoding. *Coral Reefs*.

BERRY, T. E., OSTERRIEDER, S. K., MURRAY, D. C., COGHLAN, M. L., RICHARDSON, A. J., GREALY, A. K., STAT, M., BEJDER, L. & BUNCE, M. 2017. DNA metabarcoding for diet analysis and biodiversity: A case study using the endangered Australian sea lion (*Neophoca cinerea*). *Ecol Evol,* 7**,** 5435-5453.

BERRY, T. E., SAUNDERS, B. J., COGHLAN, M. L., STAT, M., JARMAN, S., RICHARDSON, A. J., DAVIES, C. H., BERRY, O., HARVEY, E. S. & BUNCE, M. 2019. Marine environmental DNA biomonitoring reveals seasonal patterns in biodiversity and identifies ecosystem responses to anomalous climatic events. *PLOS Genetics*, 5(2). e1007943

HARDY, N., **BERRY, T.**, KELAHER, B. P., GOLDSWORTHY, S. D., BUNCE, M., COLEMAN, M. A., GILLANDERS, B. M., CONNELL, S. D., BLEWITT, M. & FIGUEIRA, W. 2017. Assessing the trophic ecology of top predators across a recolonisation frontier using DNA metabarcoding of diets. *Marine Ecology Progress Series*, 573, 237-254.

SIMPSON, T. J., DIAS, P. J., SNOW, M., MUNOZ, J. & **BERRY, T**. 2016. Real-time PCR detection of Didemnum perlucidum (Monniot, 1983) and Didemnum vexillum (Kott, 2002) in an applied routine marine biosecurity context. *Mol Ecol Resour*.

STAT, M., HUGGETT, M. J., BERNASCONI, R., DIBATTISTA, J. D., **BERRY, T. E.**, NEWMAN, S. J., HARVEY, E. S. & BUNCE, M. 2017. Ecosystem biomonitoring with eDNA: metabarcoding across the tree of life in a tropical marine environment. *Sci Rep*, 7, 12240.

6.5 Presented posters

2014—*Development of Molecular Tools for Dietary analysis*

Combined Biological Sciences Meeting (Perth)

2016—*An Ode to the Planktonic Relationships of Rottnest Island – A five-year metabarcoding study*

Society for Molecular Biology and Evolution Conference (Gold Coast)

Combined Biological Sciences Meeting (Perth)

2018—*Marine Environmental DNA reveals spatial and temporal community changes in zooplankton*

Combined Biological Sciences Meeting (Perth; -omics poster prize)

Development of a multi-assay approach for monitoring coral diversity using eDNA metabarcoding.

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ABSTRACT: Cumulative anthropogenic pressures have triggered a global decline in the health of marine ecosystems, and coral reefs, in particular, are in crisis. With climate and population-related pressures predicted to intensify in the coming decades, it is increasingly crucial to develop cost-effective and accurate monitoring tools to document changes to these important ecosystems. Environmental DNA (eDNA) coupled with metabarcoding is a powerful tool for surveying a wide variety of biota. Here, we develop a baseline eDNA toolkit targeting scleractinian corals and validate its performance in conjunction with data derived on traditional diver-based visual surveys at the Cocos (Keeling) Islands. Three assays targeting the ITS2 and 16S barcoding regions were designed, which broadly detected diversity within Scleractinia and Porifera. Our eDNA assays recovered 78 ITS2 operational taxonomic units (OTUs) from 25 scleractinian genera which is comparable to the level of diversity recorded on visual surveys (68 species from 26 genera). There were some notable differences in the species detected

using eDNA versus visual records that may relate to either misidentifications, intragenic variation, differential assay performance or cryptic species. Our data demonstrate that a multi-assay eDNA analytical approach, applied on surface water collections, represents a powerful and complementary way to survey diversity that can also reveal fine scale spatial differentiation in community composition. With further refinement and improved reference databases, we envisage eDNA to become a powerful complement to visual surveys and to play a key role in monitoring the health and diversity of complex coral reefs ecosystems.

KEY WORDS: Biodiversity, Biomonitoring, Coral reefs, Environmental DNA, scleractinia

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ORIGINAL RESEARCH

WILEY Ecology and Evolution Open Access

DNA metabarcoding for diet analysis and biodiversity: A case study using the endangered Australian sea lion (*Neophoca cinerea*)

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Abstract

The analysis of apex predator diet has the ability to deliver valuable insights into ecosystem health, and the potential impacts a predator might have on commercially relevant species. The Australian sea lion (*Neophoca cinerea*) is an endemic apex predator and one of the world's most endangered pinnipeds. Given that prey availability is vital to the survival of top predators, this study set out to understand what dietary information DNA metabarcoding could yield from 36 sea lion scats collected across 1,500 km of its distribution in southwest Western Australia. A combination of PCR assays were designed to target a variety of potential sea lion prey, including mammals, fish, crustaceans, cephalopods, and birds. Over 1.2 million metabarcodes identified six classes from three phyla, together representing over 80 taxa. The results confirm that the Australian sea lion is a wide-ranging opportunistic predator that consumes an array of mainly demersal fauna. Further, the important commercial species *Sepioteuthis australis* (southern calamari squid) and *Panulirus cygnus* (western rock lobster) were detected, but were present in <25% of samples. Some of the taxa identified, such as fish, sharks and rays, clarify previous knowledge of sea lion prey, and some, such as eel taxa and two gastropod species, represent new dietary insights. Even with modest sample sizes, a spatial analysis of taxa and operational taxonomic units found within the scat shows significant differences in diet between many of the sample locations and identifies the primary taxa that are driving this variance. This study provides new insights into the diet of this endangered predator and confirms the efficacy of DNA metabarcoding of scat as a noninvasive tool to more broadly define regional biodiversity.

KEYWORDS

apex predator, dietary scat analysis, DNA metabarcoding, *Neophoca cinerea*, next generation sequencing

1 | INTRODUCTION

The majority of marine mammals are generalist predators that consume prey from many trophic levels (Casper, Jarman, Gales, & Hindell, 2007) and therefore potentially influence the community structure of marine environments. As such, the analysis of their diet can provide the opportunity for a comprehensive assessment of the biodiversity present in marine ecosystems (Boyer, Cruickshank, & Wratten, 2015; Casper et al., 2007).

The Australian sea lion (Figure 1) is one of the rarest sea lion species in the world (Hesp et al., 2012) and Australia's only endemic pinniped species (Kirkwood & Goldsworthy, 2013; Ling, 1992). In 2015, there were an estimated 12,290–13,090 individuals remaining in the wild and of these only 16% are found in Western Australia (Goldsworthy, 2015). Australian sea lions are distributed between the Abrolhos Islands in Western Australia and The Pages in South Australia (Ling, 1992), with mostly small and widely scattered colonies, at both remote (Goldsworthy, 2015; Goldsworthy et al., 2009) and near metropolitan areas (Osterrieder, Salgado Kent, & Robinson, 2015, 2016). Despite several dietary studies (Casper et al., 2007; Gales & Cheal, 1992; Kirkwood & Goldsworthy, 2013; Ling, 1992; Peters et al., 2014), much of what this apex predator targets remains poorly defined due to the well-recognized limits of morphological identification of scat material and/or behavioral studies (Kirkwood & Goldsworthy, 2013). Such reports suggest that the Australian sea lion is a largely nocturnal forager (Kirkwood & Goldsworthy, 2013), although studies of females and pups from Kangaroo Island, South Australia, suggest that their foraging does not follow a diurnal pattern (Costa & Gales, 2003). These previous studies have also shown that sea lions prey mainly on benthic species of fish, sharks, rays, cephalopods, and crustaceans (Kirkwood & Goldsworthy, 2013); however, other evidence also suggests that they prey on rock lobster, swimming crabs, shark eggs, and penguins (McIntosh, Page, & Goldsworthy, 2006). A more recent molecular approach used bacterial cloning and Sanger sequencing of DNA to identify 23 fish and five cephalopod taxa from the scats of 12 female sea lions from two colonies in South Australia (Peters et al., 2014), finding several new taxa upon which sea lions prey.

FIGURE 1 The Australian sea lion (Neophoca cinerea) at Seal Island, Shoalwater Bay, Western Australia

Observational studies on diet in marine systems can be logistically difficult to conduct and expensive. This is especially true where the animal in question is fast, feeds underwater, and has a large foraging range, as is the case with sea lions (Kirkwood & Goldsworthy, 2013). These problems can be compounded when the study animal is reclusive and/or hunts nocturnally (such as sea lions). In contrast, the collection of sea lion scat is relatively easy as it can be collected by hand from the beaches of known sea lion haul out points. However, morphological analysis of scat has several complications. Firstly, dietary identification relies heavily on the presence of prey remnants, and prey that is relatively undigested may be over represented while highly digested prey may be missed (Boyer et al., 2015; Brown, Jarman, & Symondson, 2012; Shehzad, McCarthy, et al., 2012). Therefore, fleshy or gelatinous targets are unlikely to be detected. In the case of the sea lion, smaller cephalopod beaks and fish otoliths digest completely, or are unrecognizable, once they have passed through the digestive tract (Gales & Cheal, 1992; Peters, Ophelkeller, Bott, & Goldsworthy, 2015). This issue is partially attributed to the grinding action of large gastroliths found in the sea lions' stomach (McIntosh et al., 2006). Gastroliths are large stones that can measure up to approximately 7 cm in diameter and are swallowed by sea lions as ballast (Kirkwood & Goldsworthy, 2013). Secondly, some potential prey species, such as crustaceans, are morphologically similar to one another (Radulovici, Sainte-Marie, & Dufresne, 2009), making identification of their remains taxonomically challenging. Further, due to the increased rate of survival of cephalopod beaks in comparisons to fish otoliths, reliance on morphological analysis of sea lion scat for dietary analysis can lead to an underestimation of fish but an overestimation of cephalopods consumed (Gales & Cheal, 1992; Peters et al., 2015).

Recent advances in DNA sequencing (and analyzing) environmental samples have enhanced the capacity to identify constituents of fecal material (Pompanon et al., 2012). The use of standard DNA barcodes, PCR, and reference sequence databases facilitates the analysis of prey taxa (or their DNA) that survive in fecal material. DNA metabarcoding approaches (employing next generation sequencing, NGS), where complex mixtures of DNA are extracted and sequenced in parallel, have been successfully applied to several fecal dietary studies with promising results (Berry et al., 2015; Hibert et al., 2013; Murray et al., 2011; Quemere et al., 2013; Shehzad, Riaz, et al., 2012). One of the first studies to exploit DNA metabarcoding, investigated the diet of the Australian fur seal (*Arctocephalus pusillus*; (Deagle, Kirkwood, & Jarman, 2009) and, in a more recent study, the diets of both the Australian (*A. pusillus doriferus*) and long-nosed fur seals (*A. forsterii*) were compared (Hardy et al., In press). To date, no metabarcoding studies exist to explore the Australian sea lion diet but recently a gut microbiome study was conducted on both wild and captive populations (Delport, Power, Harcourt, Webster, & Tetu, 2016). It is suggested that this type of study could, in future, be combined with a dietary analysis to determine what impact diet has on gut flora.

Using DNA metabarcoding on 36 scat samples, this study seeks to develop and apply multi-gene metabarcoding assays for the analysis of the diet of the Australian sea lion. The purpose of the results is threefold: (1) to determine the effectiveness of DNA metabarcoding

for the dietary analysis of the Australian sea lion and the marine biodiversity that supports them, (2) to assess the predation of commercially valued fishes, and (3) to establish whether this type of study could be used to detect spatial changes in sea lion prey across the southwest of Australia. Importantly, as the Australian sea lion is an endangered species (IUCN Red List; Goldsworthy, 2015), it is of value to develop a holistic picture of what dietary options these apex predators exploit and how these differ spatially and temporally.

2 | METHODS

2.1 | Sample collection

In total, 36 fecal samples were collected in sterile containers from islands across five collection sites that stretch 1,500 km of the southwest coast of Western Australia (Figure 2; for greater detail about dates and sites see Table A1). Scat samples were preserved and stored at −20°C.

2.2 | Metabarcoding assay design

Several PCR assays were designed and/or optimized for use in DNA metabarcoding workflows including the Fish 16S, Ceph 16S, and the Crust 16S assays (Table 1). All primer sets flank hypervariable regions of the 16S rRNA gene and were designed and tested in silico using reference sequences obtained from GenBank. For the Ceph 16S assay, 27 16S sequences from different Western Australian cephalopods were analyzed in silico to identify short conserved areas of the target gene, which will amplify degraded DNA. Similarly, the Crust 16S assay was designed using 13 16S crustacean sequences including crayfish, crab, and prawn species. All newly designed primers were tested against sea lion sequences to ensure no significant amplification of host DNA. To determine the efficacy of the assays, amplifications were optimized on single-source reference tissue including some crustaceans, a cephalopod, and several species of fish (Table A2).

2.3 | DNA extraction and quantification

Scats were subsampled (100–290 mg) and the DNA was extracted using the QIAmp Stool Mini Kit (Qiagen, CA, USA), following the manufacturer's instructions but using an overnight digestion at 55°C, 0.5× InhibitEX tablet, and eluting in 50 µl of AE Buffer. Extracts were diluted (1/5 and 1/20) in order to assess assay response, and amplification efficiency and inhibition using quantitative PCR (qPCR). All qPCR reactions were carried out in 25 µl consisting of final concentrations of: 1× Taq Gold buffer (Applied Biosystems [ABI], USA), 2 mmol/L $MgCl_2$ (ABI, USA), 0.4 mg/ml BSA (Fisher Biotec, Australia), 0.25 mmol/L dNTPs (Astral Scientific, Australia), 0.4 µmol/L each of forward and reverse primers (Integrated DNA Technologies, Australia), 0.6 µl of 1/10,000 SYBR Green dye (Life Technologies, USA), 1 U of Taq polymerase Gold (ABI, USA), 2 µl of DNA, and made to volume with ultrapure water.

Each qPCR was run on a Step-ONE qPCR thermocycler (ABI, USA) under the following conditions: 95°C for 5 min, followed by 50 cycles of 95°C for 30 s, 54–58°C for 30 s (the annealing temperature of each primer set is represented in Table 1) and 72°C for 45 s and a final extension of 10 min at 72°C. Where qPCR of an extract produced results in response to an assay, the DNA dilution with the highest relative proportion of starting template that showed uninhibited

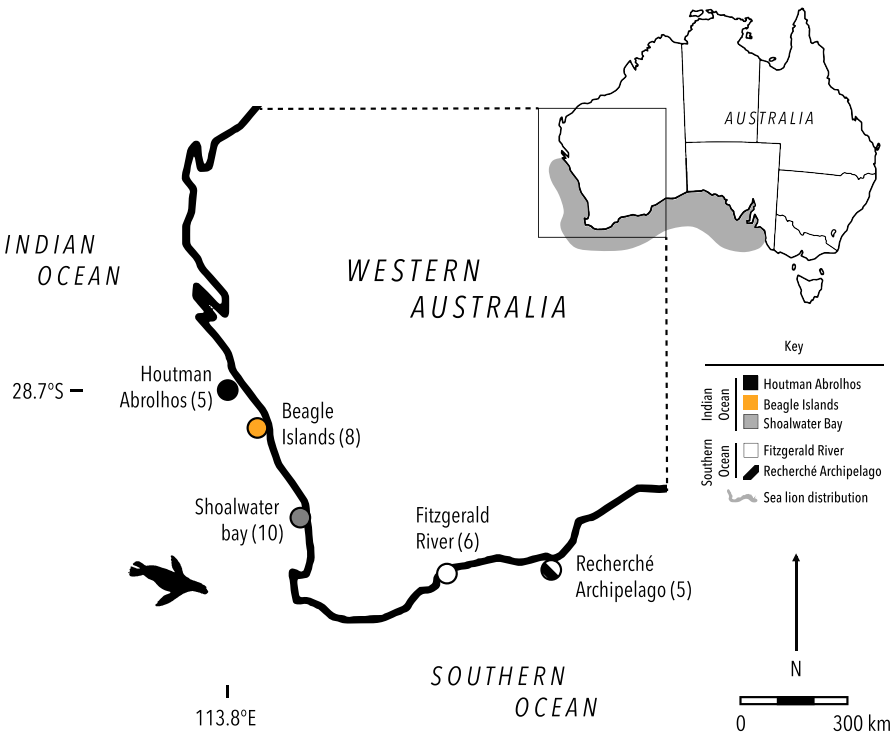

FIGURE 2 Sampling sites for metabarcoding study; Map of Australia, with inset showing southern Western Australian sampling sites (number of scats in brackets). The shaded areas denote the range of the Australian sea lion across Australia and within Western Australia

TABLE 1 Metabarcoding PCR assays and the primer sets used for dietary analysis of *Neophoca cinerea* scat

PCR assay	Primer set used	Target Taxa	Gene	Primer sequence	Amplicon length (bp)	Reference	Assay T_m (°C)
Bird 12S	12Sa (F)	Birds	12S rRNA	5′ CTGGGATTAGATACCCCACTAT 3′	~230	Cooper (1994)	57
	12Sh (R)			5′ CCTTGACCTGTCTTGTTAGC 3′			
Fish 16S	Fish16sF/D	Fish	16S rRNA	5′ GACCCTATGGAGCTTTAGAC 3′	~200	F-This study	54
	16s2R (degenerate)			5′ CGCTGTTATCCCTADRGTAACT 3′		R-Deagle et al. (2007)	
Plank COI	(Plank) Minibar-Mod-F	Plankton	COI	5′ TCCACTAATCACAAAGAYATYGGYAC 3′	~127	Berry et al. (2015)	52
	(Plank) Minibar-Mod-R			5′ AGAAAATCATAATRAANGCRTGNGC 3′			
Ceph 16S	Ceph16S1_F(deg)	Cephalopods	16S rRNA	5′ GACGAGAAGACCCTADTGAGC 3′	~200	F- Peters et al. (2014)	55
	Ceph16SR_Short			5′ CCAACATCGAGGTCGCAATC 3′		R-This study	
Crust 16S	Crust16S_F(short)	Crustaceans	16S rRNA	5′ GGGACGATAAGACCCTATA 3′	~170	This study	51
	Crust16S_R(short)			5′ ATTACGCTGTTATCCCTAAAG 3′			
Mam 16S	16Smam1 (F)	Mammals	16S rRNA	5′ CGGTTGGGGTGACCTCGGA 3′	~90	Taylor (1996)	57
	16Smam2 (R)			5′ GCTGTTATCCCTAGGGTAACT 3′			
S_Ceph 16S	S_Cephalopoda_F	Cephalopods	16S rRNA	5′ GCTRGAATGAATGGTTTGAC 3′	~70	Peters et al. (2014)	50
	S_Cephalopoda_R			5′ TCAWTAGGGTCTTCTCGTCC 3′			

"F" refers to the forward primer; "R" refers to the reverse primer.

amplification (determined by qPCR C_T values) was selected for subsequent metabarcoding using assay-specific fusion tagged primers (The number of PCR-positive samples from each site and assay are shown in Table A3). The optimization of input DNA in amplicon sequencing workflows has been shown previously to benefit the sensitivity, reproducibility, and quality of metabarcoding data (Murray, Coghlan, & Bunce, 2015).

2.4 | Library build and sequencing

Fusion tagged primers are gene-specific primers which also incorporate MID (Multiplex IDentifier) tags of six to eight base pairs in length, and the appropriate Illumina/454 adaptor sequences. Unique combinations of these MID tags were assigned to each individual DNA extract to allow for the assignment of sequences to a sample post-sequencing of pooled samples. To minimize cross-contamination (in highly sensitive NGS workflows), no primer-MID combination had been previously used, nor were combinations reused. Fusion PCR reactions were performed on DNA extracts (appropriate dilution determined by qPCR) in duplicate, and thermocycling conditions were used as described above. Tagged amplicons were purified using the Agencourt™ AMPure™ (Beckman Coulter Genomics, MA, USA) XP Bead PCR Purification kit as per the manufacturer's instructions, with the addition of a five-minute incubation prior to elution at room temperature. The size and concentration of amplicons were estimated by electrophoresis on a 2% agarose gel stained with GelRed (Fisher Biotec, Australia), followed by visualization under UV light using a Bio-Rad transilluminator.

Amplicons were combined in approximately equimolar concentrations to produce a single DNA library of all extracts for sequencing. The resultant library was purified as described above and quantified alongside a set of standard synthetic oligonucleotides of known molarity (Bunce, Oskam, & Allentoft, 2012) via qPCR, prior to sequencing (95°C for 5 min followed by 40 cycles of 95°C for 30 s and 60°C for 45 s). For the Mam 16S and Bird 12S assays, all sequencing was performed on Roche's 454 GS Junior (Lib A chemistry). For the remainder of the assays, sequencing was achieved using Illumina's MiSeq® (300 cycle, version 2 reagent kit and Nano flow cell), following manufacturers protocols.

2.5 | Data filtering and bioinformatics

Sequences were assigned to samples based on their MID tag using Geneious v.R8 (Kearse et al., 2012). As a method for quality control, only amplicons that contained a 100% nucleotide match to the MID, gene-specific primer, and sequencing adapter regions were kept for further analysis (the number of reads passing this filter for each assay and per site is shown in Table A4). Adaptor/primer regions were removed, and the remaining amplicons were filtered using USEARCH's fastq filter with a maximum error of 0.5 (Edgar, 2010). The sequences were then separated into groups of unique sequences (these data are available for download on Data Dryad, https://doi.org/10.5061/dryad.rd748). Groups with sequence numbers of <1% of the total number of unique sequences detected in the sample were discarded in order to remove low-abundant and potentially erroneous sequences (i.e., sequencing error and chimeras). Amplicons

passing quality filtering were searched against the National Center for Biotechnology Information's (NCBI) GenBank nucleotide database (April 29 2015; Benson et al., 2015) using BLASTn (Basic Local Alignment Search Tool; Altschul, Gish, Miller, Myers, & Lipman, 1990) with the default parameters and a reward of 1. BLAST output files were imported into MEGAN (METaGenome ANalyzer; Huson, Mitra, Ruscheweyh, Weber, & Schuster, 2011) and visualized against the NCBI taxonomic framework using the LCA parameters: reporting of all reads, min bitscore 65.0, and reports limited to top 5% matches. Assignment of sequences to taxa was only considered where a match was made across the entire length of the query. Where further information was required regarding the habitat and commercialization of a species, the Atlas of Living Australia (2016) and FishBase (Froese & Pauly, 2016) were consulted (the number of reads assigned for each site and assay is shown in Table A4).

2.6 | Operational taxonomic unit analysis

The operational taxonomic unit (OTU) analysis was performed using USEARCH (Edgar, 2010). Sequences were grouped into clusters (OTUs) using a 97% similarity threshold. The process also removed any chimeras, as well as clusters with a sequence abundance below 0.75% of the total number of unique sequences detected within the sample. Empirically these thresholds retained the sensitivity of the metabarcoding assays but removed low abundance OTUs that may be sequencing/PCR artifacts.

2.7 | Statistical analysis

Despite the modest number of samples and sites, and the issues involving sampling times of the year, a statistical analysis was explored. Accordingly, a Jaccard dissimilarity index of the presence/absence data was performed in R (R Core Team, 2015) using the Vegan (Oksanen et al., 2016) and labdsv (Indval; Roberts, 2016) packages. A nested nonparametric (permutational) multivariate analysis of variance (adonis) was used to determine whether sea lion diet differed significantly between the five sampling areas nested within the Southern and Indian Oceans. A pairwise adonis with Holm correction (McLaughlin & Sainani, 2014) was also undertaken to ascertain the contribution of each site to the differences seen. The relationship of sampling sites was visualized using a nonmetric multidimensional scaling (nMDS). Finally, an estimate of indicator value (indval; Dufrêne & Legendre (1997)) was calculated to determine which taxa significantly influenced any differences observed in sea lion diet between oceans and among sites within each ocean. While it was tempting to investigate the relative abundance of NGS reads (within a PCR assay), the value of extracting quantitative data is questionable and unreliable. This is due to the variability in digestion rate and prey biomass, primer bias, mitochondrial molarity, and lack of conversion factors (Deagle et al., 2005; Thomas, Jarman, Haman, Trites, & Deagle, 2014). Accordingly, analyses were restricted to the presence/absence data.

3 | RESULTS AND DISCUSSION

3.1 | Overview of the results

The Mam 16S assay was used first to test whether the scat collected originated from an Australian sea lion. The remainder of the metabarcoding assays were used to determine the prey diversity found within the sea lion scats from each site. The taxa found belonged to six classes (Figure 3) from three phyla, representing over 20 orders and almost 40 families of prey.

The Mam 16S assay confirmed that 34 of the 36 beach-collected samples originated from Australian sea lions (100% matches to reference *Neophoca cinerea* DNA sequences), many of these were later confirmed by the Plank COI assay. Of the two negative samples, one contained large amounts of human DNA while the other contained DNA that was amplified by the bird-specific primers, potentially identifying the originator of the scat as *Pellicanus conspicillatus* (Australian pelican). These two samples were excluded from further analysis.

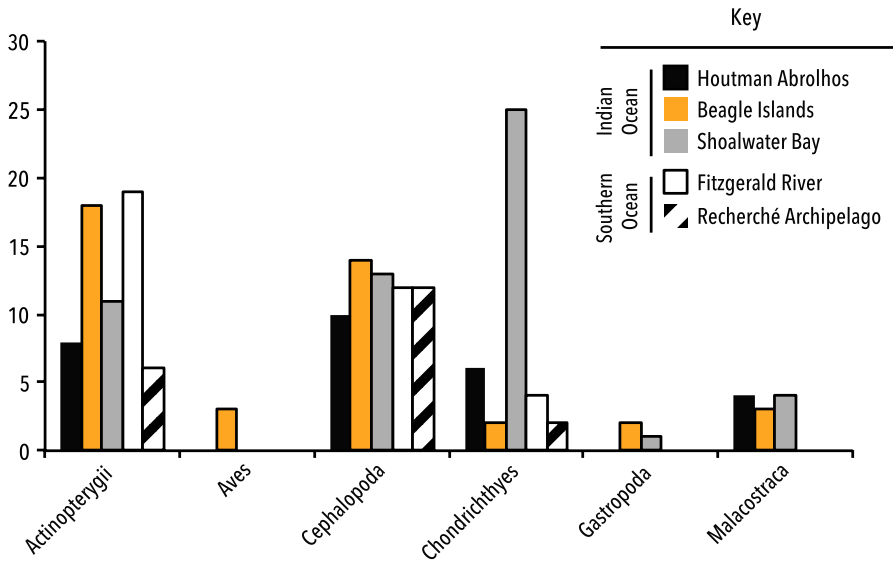

FIGURE 3 Sea lion diet: Classes of Taxa detected across the five WA study sites. The frequency a class of prey taxa was identified at each site using metabarcoding

The nonmammalian metabarcoding assays were designed to characterize fish, crustacean, and cephalopod prey in these environmental samples. It is suggested that these assays will be useful for future metabarcoding studies on marine substrates such as scat, water, sediment, plankton tows, and gut contents.

Overall the multigene metabarcoding generated in excess of 1.2 million NGS reads, which were converted to the presence/absence data. These assays revealed (Figure 3) that while the majority of the sea lion samples (~68%) contained both ray-finned fishes (Actinopterygii) and cephalopods (Cephalopoda), many sharks and rays (Chondrichthyes; ~22%) were also detected. This is especially true for those samples from Shoalwater Bay where Chondrichthyes made up the largest proportion of prey (~46%). The least common taxa were Aves and Gastropoda with only three detections each across the five sites. Table A3 shows the number of samples from each site that responded to each assay.

These findings are broadly consistent with the literature, although Kirkwood and Goldsworthy (2013) identify cephalopods as the top four sea lion prey items, followed by sharks and rays, lobsters and finally four species of ray-finned fishes. However, their study concentrates on sea lions from South Australian waters where species composition will differ to those in the WA sites studied here. The Indian Ocean sites also contained 11 incidences of malacostracans (a class of crustaceans that includes crayfish and shrimp) and three of gastropods (a class of molluscs which contains bivalves), whereas these taxa were absent from the Southern Ocean sites.

The majority of the identified prey are benthic and are usually found at depths ≤150 m and most are found at <80 m. This finding concurs with studies that suggest the maximum foraging diving depth for an adult male sea lion is 150 m (Kirkwood & Goldsworthy, 2013).

3.2 | Sea lion diet—Fish detections

Fish sequences were detected using both the Fish 16S and the Plank COI assays. Together, the two metabarcoding assays identified 47 Actinopterygii—36 of which were assigned to a genus or species level—and 17 Chondrichthyes—13 of which were ascribed to a genus or species level (Tables A5 and A6).

While there was some redundancy in the two assays that target fish, typically they detected different taxa; only five of the taxa were detected by both assays (Table A5). The Fish 16S assay detected 72% of the ray-finned fishes compared with the Plank COI assay, which detected 38% of the ray-finned fishes identified. For the cartilaginous fish, this trend was reversed, with the Fish 16S assay detecting 41% of the taxa identified and the Plank COI assay yielding 71%; only one genus (*Mustelus*) was detected by both assays (Table A6). These results demonstrate that, even with broad-spectrum ("universal") PCR assays, important species are still missed, and that when metabarcoding assays are used in combination, they yield far more information about the biodiversity of environmental samples. This is because the biotic "background" will vary between sites/samples and "generic" primers will exhibit sample dependent bias, where, due to primer binding variation, one group of taxa will preferentially amplify over another where they are both present in the sample (Pompanon et al., 2012). These biases are manifest further when samples are in low copy number and/or inhibited (Murray et al., 2015).

Comparing sites, Perciformes were detected in all five samples from Houtman Abrolhos and the Beagle Islands, but were only detected in four of the six samples from Fitzgerald River, and were detected even less frequently in samples from Shoalwater Bay and Recherche Archipelago (Figure 4a). The order Perciformes contains a large variety of perch-like fish including wrasse, parrotfish, goatfish, and damselfish. Fifteen taxa from this order were detected overall, with the vast majority of these identified from the Beagle Islands samples. The likely reason for this is that while Perciformes are found in all areas of southern Western Australia, the majority of those species detected in the sea lion scat are mainly found in the Indian Ocean. An example of this is *Pomacanthus semicirculatus*, which has only been recorded in northern waters of Australia (ALA, 2016). There is also a climatic shift from the Indian (warmer) and Southern Oceans (cooler) that may result in differences in prey species for sea lions. Tetraodontiformes, which includes the family Monacanthidae (leatherjackets), also seems to be favored across three sites (Beagle Island, Shoalwater Bay, and Fitzgerald River; Figure 4a). All these findings are in line with those of Peters et al. (2014), who also identified wrasse, goatfish, and leatherjackets as important prey for sea lions.

Of note is the detection of eels (Anguilliformes) as prey, by both the Fish and Plank COI assays. The species detected include the highfin moray (*Gymnothorax pseudothyrsoideus*), conger eels (*Conger* and *Gnathophis*), and unknown species of knot eels (in the Muraenidae family). The consumption of eels by the sea lions has not previously been reported, and yet the frequency of occurrence (eight samples across all five sites) suggests this is a regular component of sea lion diet.

In contrast to the other sites, a large proportion of sharks and rays are consumed by sea lions at Shoalwater Bay (Figure 4b). Each of the ten samples from Shoalwater contained prey from all five orders of Chondrochthyes detected, including stingarees (Urolophidae) and wobbegongs (Orectolobidae). Even in the Houtman Abrolhos Islands, four of the five samples produced sequences matching wobbegongs (Orectolobidae). While the Australian sea lion is known to eat sharks and rays (Kirkwood & Goldsworthy, 2013; Ling, 1992), it is suggested that many of the taxa identified here are previously unrecognized as sea lion prey.

The vast majority of the fishes detected in this study are classified as demersal or benthic and are found associated with reefs, seagrass, and the rocky and sandy bottoms of the continental shelf. This finding is consistent with current knowledge that describes the Australian sea lion as diving for its prey and being a principally benthic feeder (Gales & Cheal, 1992; Hesp et al., 2012; Kirkwood & Goldsworthy, 2013).

3.3 | Sea Lion diet—Fish OTUs

There is a growing trend to move to taxonomic-independent methods such as OTUs when describing genetic diversity in marine environments using metabarcoding data. This type of analysis allows for examination of all the available genetic diversity in metabarcoding data without the constraints of a frequently imprecise (and constantly

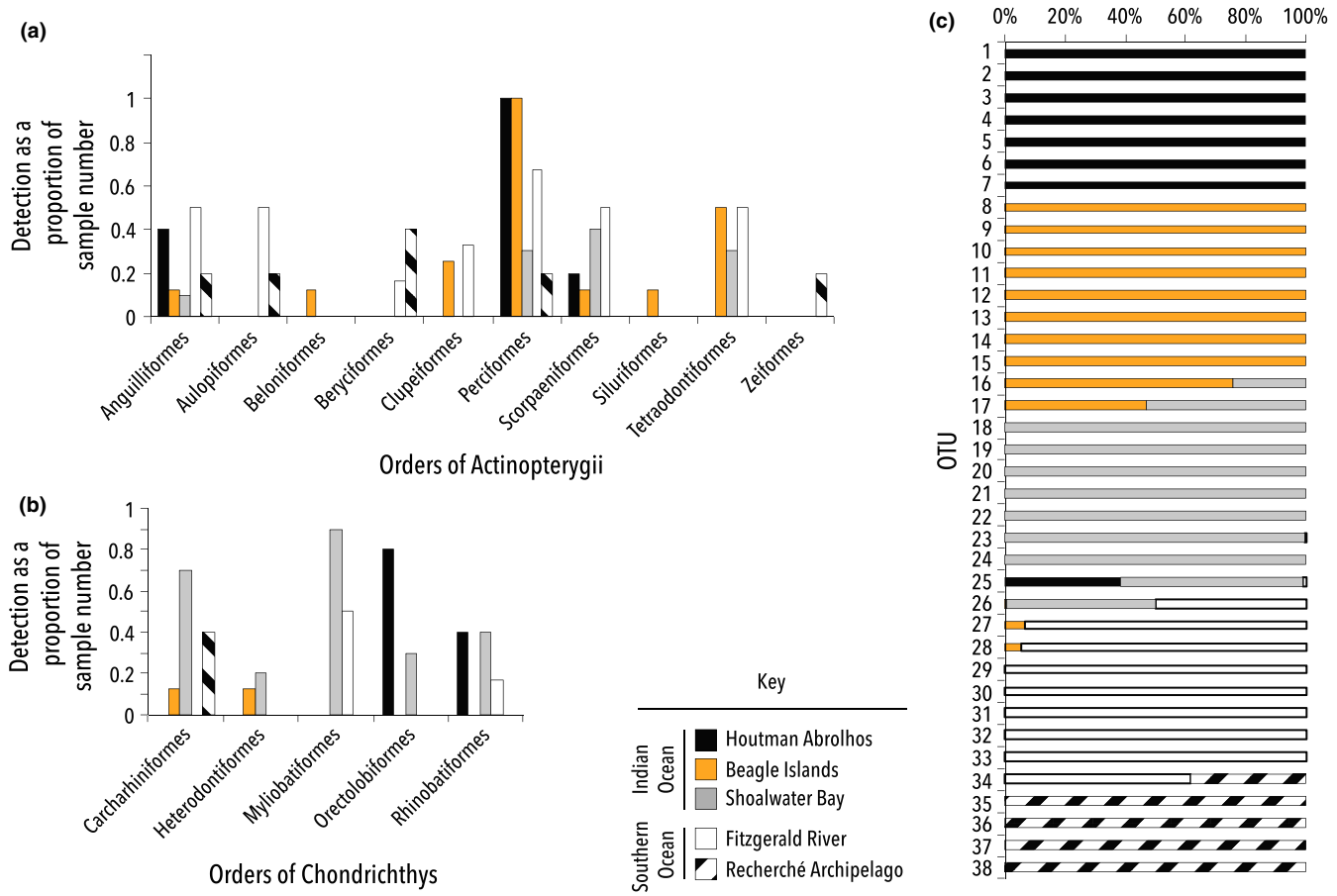

FIGURE 4 Metabarcoding of sea lion diet analyzed using ordinal and operational taxonomic unit (OTU) assignments. The number of times an order within (a) Actinopterygii and (b) Chondrichthyes was detected at each site as a proportion of the number of scat samples taken from each sample location. The OTU analysis of the Fish16S assay (c) demonstrates clear divisions between the genetic diversity of fish in the sample sites and between those samples sourced in the Indian Ocean compared with those from the Southern Ocean. The data used for (c) can be found in Table A9

evolving) taxonomic framework, coupled with an often-incomplete collection of reference DNA barcodes.

Given the large number of fish taxa detected (Tables A5 and A6) using the Fish 16S metabarcoding assay, we analyzed the estimated genetic diversity of fish between sites using OTUs. After filtering, a total of 38 OTUs (Table A9; at 97% clustering) were identified from the 34 samples across the five study sites. Clear differences in regionality of fish diversity among sites were observed, with only seven of the 38 (~18%) OTUs shared across two or more sites (Figure 4c). When these sites were grouped by ocean (i.e., Southern or Indian Ocean), the division in genetic diversity was even more obvious, with only three of 38 (~8%) OTUs shared across the two oceans (Figure 4c).

From autumn to early spring (April to October), the Leeuwin Current (LC) brings warmer waters than would usually be found at these latitudes to the west coast of southern Western Australia (as well as tropical fish and invertebrates; Pearce & Feng, 2013), with the result that water temperatures are maintained at a warmer level during winter. While this current continues around to the southern coastline, it is supplemented by currents from subantarctic waters (Cresswell & Domingues, 2009), resulting in cooler environments in the Southern Ocean. Thus, the clear genetic distinction in the Fish OTUs between the oceans is likely attributable to these differences in the habitats; although we cannot rule out that temporal differences have also contributed.

3.4 | Sea lion diet—Cephalopods and gastropods

Invertebrates, especially octopus, squid, and cuttlefish, are thought to make up a large proportion of the diet of the sea lions (Hesp et al., 2012; Kirkwood & Goldsworthy, 2013; McIntosh et al., 2006; Peters et al., 2014), but the actual invertebrate prey species remain largely unknown. The Plank COI, S_Ceph 16S, and Ceph 16S metabarcoding assays identified 14 invertebrate taxa, with 11 identified to a genus or species level (Table A7). However, many of the octopus species nominally identified have not previously been described in the collection area (those not known in Australia were assigned to higher taxa). This may be because the S_Ceph primer set target is a small amplicon (~70 bp), and therefore, one erroneous base, coupled with possible low interspecific variation at this locus, could result in erroneous assignments. The other possible reason is the relatively poor representation of the class on Genbank (of the taxa searched for in this study less than 75% had a 16S mtDNA sequence deposited in the

database). However, as reference databases improve at widely utilized metabarcoding targets, so will our ability to make more robust taxonomic identifications.

Interestingly, the Plank COI assay also detected some cephalopods that provided additional support for taxa detected by both the Ceph and S_Ceph primers, in particular *Octopus* and *Sepia apama*. These two taxa were detected in 21 and 25 samples, respectively, and across all sites (Figure 5).

Of particular interest is the detection of the southern calamari squid (*S. australis*, order Teuthida), an important commercial species in Australia. While this species was detected in samples across four of the five sites (Figure 5), it was detected in less than a quarter of all samples (~18%), and in these samples, this was not the only prey revealed. This may indicate that the sea lions prefer octopus and giant cuttlefish to calamari, or it may suggest that the southern calamari squid is less abundant in the areas sampled. This latter possibility is perhaps more likely, as the occurrence records from the Atlas of Living Australia (2016) shows a decrease in the incidence of squid sightings west of the border with South Australia. Furthermore, in a South Australian sea lion study, Peters et al. (2014) also documented that *S. australis* is common prey.

The number of gastropod species detected was limited (Table A7) and these taxa have, to our knowledge, not been identified previously as potential sea lion prey. *Haliotis diversicolor* (many-colored abalone) is found in the area where it was detected (ALA, 2016) and while *Stomatella impertusa* (False ear shell) was represented by only a few sequences in one sample, it does reside in Australian waters and the Genbank record had a 100% match with the queried sequence. Despite observing these taxa in more than one scat, it is difficult to exclude the possibility that the observations may be a consequence of secondary predation (the carryover of DNA from the gut of ingested prey species).

3.5 | Sea lion diet—Crustaceans and birds

Crustaceans, including rock lobsters and swimming crabs, are noted as common prey of the Australian sea lion in South Australia (Kirkwood & Goldsworthy, 2013; McIntosh et al., 2006). The newly developed Crust16S assay detected five taxa, three to species level (Table A8). The results confirm that the Australian sea lion does prey on the commercially important western rock lobster (*Panulirus cygnus*), which was detected in six samples across all three sites in the Indian Ocean. This assay also detected a species of swimming crab (*Thalamita danae*) in a sample from the Houtman Abrolhos Islands; the only site where it is likely to be found (ALA, 2016).

The site at Shoalwater Bay is close to Penguin Island, which is home to a colony of little penguins (*Eudyptula minor*), a bird that is reported to be preyed upon by sea lions (McIntosh et al., 2006); as such, all samples were screened using the Bird 12S assay (which has been confirmed to detect *E. minor* in silico and in vivo), but no penguins were detected. However, we did detect the presence of one bird, a pied cormorant (*Phalocrocorax varius*), in three samples from Beagle Islands, which was also confirmed using the Plank COI assay. One of these samples also contained DNA from a bridled tern (*Onychoprion anaethetus*). While environmental contamination (e.g., sand on the beach, which was excluded as far as practicable) cannot be ruled out to explain the presence of both of these birds, neither can predation. Neither species of these birds has previously been documented as potential prey for Australian sea lions, but both are known to sit on the surface of the water (the pied cormorant also dives below the surface) and are thus susceptible to ambush predation from below.

Neither birds nor crustaceans were detected in the scats taken from the Southern Ocean sites. This may be because many of the crustaceans detected in the Indian Ocean are not known in the Southern Ocean, and while there are decapods in the Southern Ocean, they are not as prevalent as in other areas of Australia (ALA, 2016). However, as neither birds nor crustaceans appeared to make up a large proportion of the diet of the Indian Ocean sea lions, their absence in the diet of the Southern Ocean sea lions may be attributed merely to limited sample numbers, or prey preference at the time of sampling.

3.6 | Spatial differences in sea lion diet

The nested PERMANOVA (adonis) analysis showed that taxa preyed upon by sea lions were significantly different among Sites ($p < .01$) and between the Indian and Southern Ocean ($p < .0001$). A metaMDS plot (stress = 0.1595043) was used to visualize the differences in taxonomic assemblages among the five sampling sites and between the

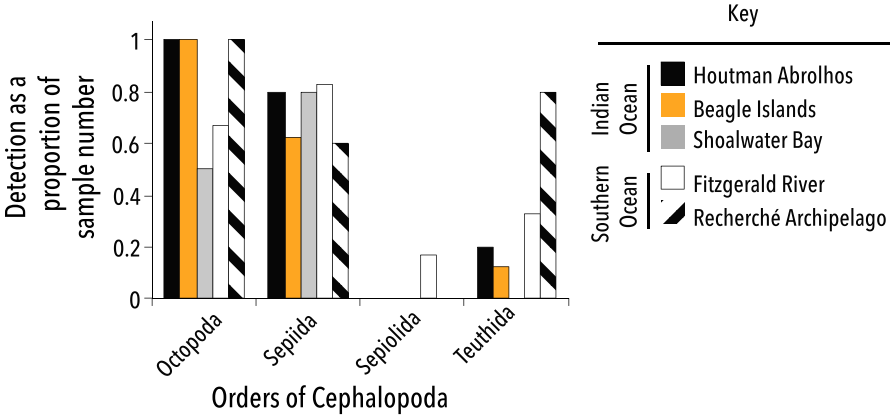

FIGURE 5 Sea lion diet: Orders of Cephalopod detected. The number of times an order within Cephalopoda was detected at each site as a proportion of the scat samples taken from each area. Data were obtained using the Ceph 16S, S_Ceph 16S, and Plank COI assays

Indian and Southern Oceans (Figure 6). There was obvious clustering for the oceanic data; however, the distinctions between the individual sites were not as clear.

To investigate this, and despite the modest sample size, further PERMANOVA (adonis) analyses were conducted to explore potential differences within each ocean. These identified an overall significant difference between the three Indian Ocean sites ($p < .007$) but no significant variance among the two Southern Ocean sites. Subsequently, a pairwise adonis was used investigate which Indian Ocean sites were different; this revealed that the only significant difference was between Houtman Abrolhos and Beagle Islands ($p < .05$).

To determine which taxa contributed to the significant differences in the PERMANOVA, indicator values analysis (indval) was performed. An indval analysis enables the taxa responsible for the regionality in the data to be discerned. While the 34 scats analyzed here are somewhat underpowered, the analysis is valuable due to the identification of taxa that drive the spatial patterns in the data. The indval analysis executed on the total metabarcoding dataset identified nine primary taxa that drive the variation in sea lion diet among sites ($p = .005–.04$), and three primary taxa that drive the differences in taxonomic assemblage observed between the Indian and Southern Ocean ($p = .002–.04$; Figure 7).

Given that birds and crustacea were only detected in the Indian Ocean, it may have been expected that these taxa would drive differences between the two oceans. However, this is not the case; in the Indian Ocean, it is *Octopus tetricus* that is flagged as a key indicator species and in the Southern Ocean it is fish, Aulopidae (threadsails) and in particular *Aulopus purpurissatus* (sergeant baker).

In the site indval analysis, Beagle Islands had four of the nine key indicator species (a bird (*P. varius*), some Actinopterygii (Monacanthidae and *Siganus*), and a species of *Octopus*. This is in keeping with the adonis analyses above, which showed Beagle Islands were significantly different from each of the other sites. Indicator taxa characterizing Shoalwater are predominantly carpet sharks, Orectolobidae, and *Aulohalaelurus labiosus*. Carpet sharks (Orectolobidae and *Orectolobus*) are also the key indicator species for the Houtman Abrolhos Islands.

Actinopterygii are the key indicator species in the Southern Ocean sites. Aulopidae are notable taxa at both Recherche and Fitzgerald, which is unsurprising as it was flagged as key indicator for differences found between the two oceans (Figure 7). In Recherche Archipelago, *Centroberyx gerrardi* (red snapper), a commercial species, was also identified as an indicator species; although it was only found in two of the five samples taken from the area.

4 | CONCLUSION

This was the first attempt to investigate and describe the diet of the Australian sea lion by DNA metabarcoding of scats. Despite the relatively small number of scats analyzed here ($n = 34$), the results demonstrate the sensitivity of the approach to identify previously unrecorded

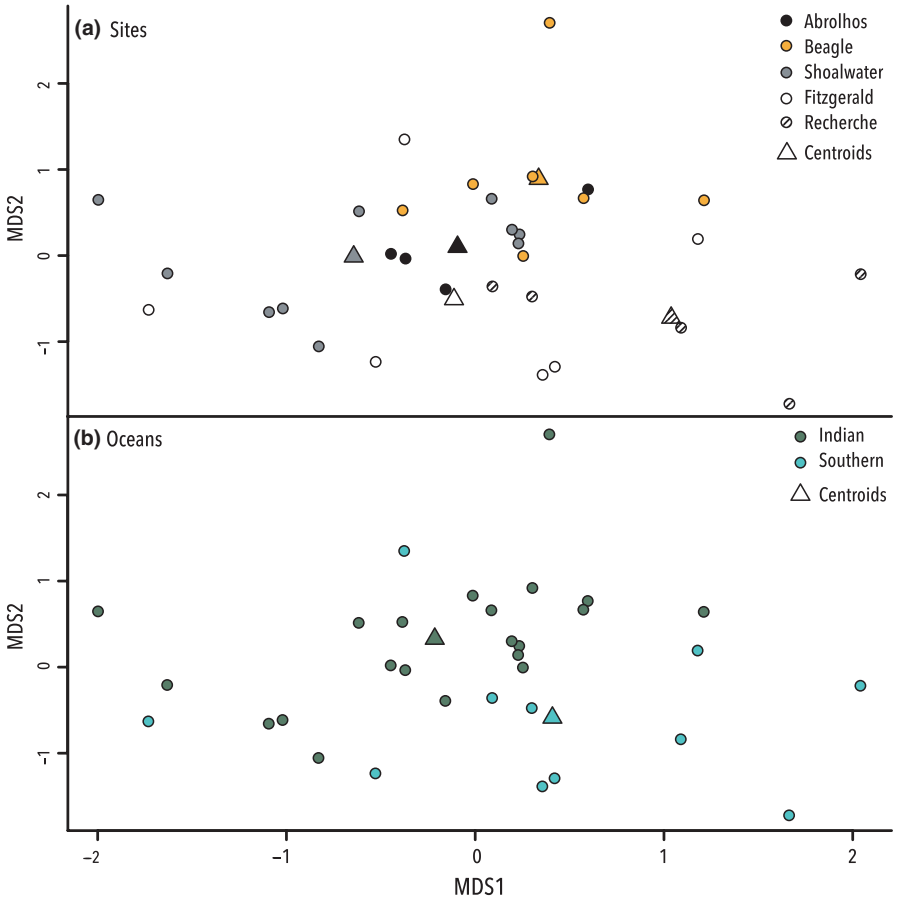

FIGURE 6 Multivariate analysis of all metabarcoding data assigned a taxonomic rank. (a) metaMDS plot comparing A taxa from the different sites of collection, and (b) the dietary differences between the sea lions of the Southern and Indian Oceans, centroids are marked with a triangles

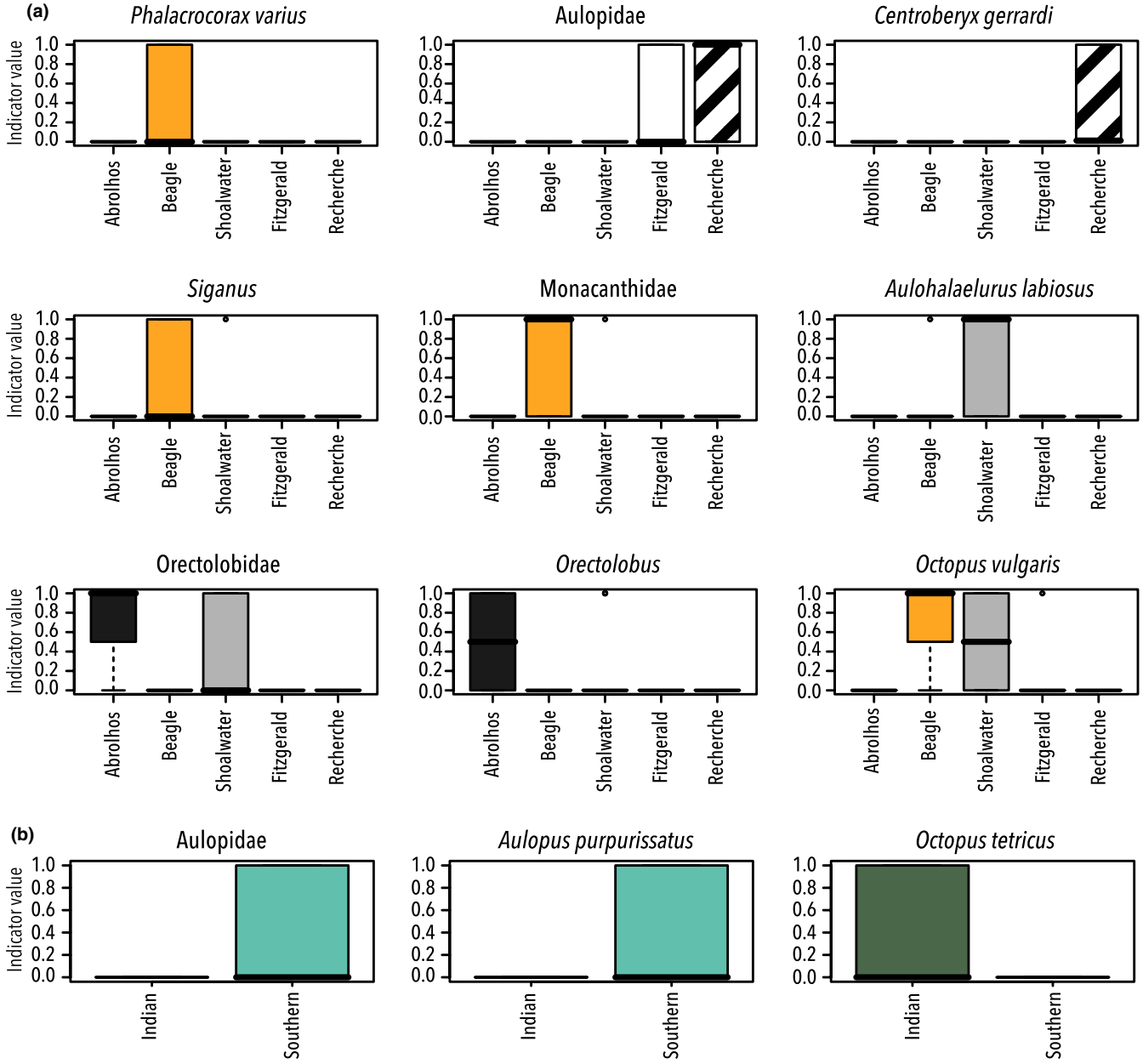

FIGURE 7 Indicator species analysis. Indval results from the total metabarcoding dataset showing the taxa characterizing each area and thus driving variations in sea lion diet between (a) sites, (b) oceans (all *p* values <.05)

species such as eels, gastropods, and the frequency of sharks and rays in the diet. Importantly, metabarcoding offers a different method allowing identification taxa that are either difficult to detect through morphological analysis of feces or through direct observational studies. This study, like previous dietary studies using metabarcoding, have been somewhat hampered by lack of reference barcodes, but despite this limitation, the dietary audit presented here presents significant insight into the prey of this apex predator. Significantly, the comprehensiveness of these datasets will improve with time, and environmental data, such as generated here, can be re-analyzed. Finally, the data gathered from the scat of this endangered apex predator demonstrate that DNA metabarcoding is a relatively simple and noninvasive way to both monitor the sea lions' diet and to provide valuable insights into the regional biodiversity of our oceans. It is foreseen that the expansion of this type of project both temporally and spatially can only add to the information gathered presented here.

Less than half of the marine species detected in this dietary study are classified as commercial species (ALA, 2016; Fishbase (Froese & Pauly, 2016)). While it is clear the sea lions are preying on some commercial species (such as the commercially important western rock lobster, *P. cygnus*, and southern calamari squid, *S. australis*), sea lions are taking a large variety of prey and no particular commercial species seems to dominate their diet. The diversity of taxa exploited by the Australian sea lion between oceans, sites, and even between samples supports the notion that Australian sea lions are opportunistic feeders. This bodes well for the survival of this protected species,

as (providing its core habitats are preserved), its mode of feeding makes it more likely to adapt its diet to changes in the surrounding biodiversity.

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CONFLICT OF INTEREST

None declared.

REFERENCES

ALA (2016). *Atlas of Living Australia*. Retrieved from http://www.ala.org.au

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*, 403–410.

Benson, D. A., Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Sayers, E. W. (2015). GenBank. *Nucleic Acids Research*, *43*(Database issue), D30–D35. http://doi.org/10.1093/nar/gku1216.

Berry, O., Bulman, C., Bunce, M., Coghlan, M., Murray, D. C., & Ward, R. D. (2015). Comparison of morphological and DNA metabarcoding analyses of diets in exploited marine fishes. *Marine Ecology Progress Series*, *540*, 167–181.

Boyer, S., Cruickshank, R. H., & Wratten, S. D. (2015). Faeces of generalist predators as 'biodiversity capsules': A new tool for biodiversity assessment in remote and inaccessible habitats. *Food Webs*, *3*, 1–6.

Brown, D. S., Jarman, S. N., & Symondson, W. O. C. (2012). Pyrosequencing of prey DNA in reptile faeces: Analysis of earthworm consumption by slow worms. *Molecular Ecology Resources*, *12*, 259–266.

Bunce, M., Oskam, C., & Allentoft, M. (2012). Quantitative real-time PCR in aDNA research. In B. Shapiro & M. Hofreiter (Eds.), *Ancient DNA: Methods and protocols, methods in molecular biology* (pp. 121–132). New York City, New York: Springer Science+Business Media.

Casper, R. M., Jarman, S. N., Gales, N. J., & Hindell, M. A. (2007). Combining DNA and morphological analyses of faecal samples improves insight into trophic interactions: A case study using a generalist predator. *Marine Biology*, *152*, 815–825.

Cooper, A. (1994). *DNA from museum specimens*. New York, NY: Springer.

Costa, D. P., & Gales, N. J. (2003). Energetics of a benthic diver: Seasonal foraging ecology of the Australian sea lion, *Neophoca Cinerea*. *Ecological Monographs*, *73*, 27–43.

Cresswell, G., & Domingues, C. M. (2009). Leeuwin current A2. In J. H. Steele (Ed.), *Encyclopedia of ocean sciences* (2nd ed., pp. 444–454). Oxford, UK: Academic Press.

Deagle, B. E., Gales, N. J., Evans, K., Jarman, S. N., Robinson, S., Trebilco, R., & Hindell, M. A. (2007). Studying seabird diet through genetic analysis of faeces: A case study on macaroni penguins (*Eudyptes chrysolophus*). *PLoS One*, *2*, e831.

Deagle, B. E., Kirkwood, R., & Jarman, S. N. (2009). Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces. *Molecular Ecology*, *18*, 2022–2038.

Deagle, B. E., Tollit, D. J., Jarman, S. N., Hindell, M. A., Trites, A. W., & Gales, N. J. (2005). Molecular scatology as a tool to study diet: Analysis of prey DNA in scats from captive Steller sea lions. *Molecular Ecology*, *14*, 1831–1842.

Delport, T. C., Power, M. L., Harcourt, R. G., Webster, K. N., & Tetu, S. G. (2016). Colony location and captivity influence the gut microbial community composition of the Australian sea lion (*Neophoca cinerea*). *Applied and Environmental Microbiology*, *82*, 3440–3449.

Dufrêne, M., & Legendre, P. (1997). Species assemblages and indicator species: the need for a flexible asymmetrical approach. *Ecological Monographs*, *67*, 345–366.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*, 2460–2461.

Froese, R., & Pauly, D. (2016). *Fishbase*. World Wide Web electronic publication. Retrieved from http://www.fishbase.org

Gales, N. J., & Cheal, A. J. (1992). Estimating diet composition of the Australian sea-lion (*Neophoca-Cinerea*) from scat analysis – An unreliable technique. *Wildlife Research*, *19*, 447–456.

Goldsworthy, S. D. (2015). *Neophoca cinerea*, *Australian Sea Lion*, *The IUCN Red List of Threatened Species*. https://doi.org/10.2305/iucn.uk.2015-2.rlts.t14549a45228341.en

Goldsworthy, S., McKenzie, J., Shaughnessy, P., McIntosh, R., Page, B., & Campbell, R. (2009). *An update of the report: Understanding the impediments to the growth of Australian sea lion populations*. SARDI research report series.

Hardy, N., Berry, T., Kelaher, B. P., Goldsworthy, S. D., Bunce, M., Coleman, M. A., … Figueira, W. (In press). Assessing the trophic ecology of top predators across a recolonisation frontier using DNA metabarcoding of diets. *Marine Ecology Progress Series*, https://doi.org/10.3354/meps12165

Hesp, S. A., Tweedley, J. R., McAuley, R., Tink, C. J., Campbell, R. A., Chuwen, B. M., & Hall, N. G. (2012). *Informing tisk assessment through estimating interaction rates between Australian sea lions and Western Australia's temperate demersal gillnet fisheries*, *Fisheries Research and Development Corporation Report*. Murdoch: Centre for Fish and Fisheries Research, Murdoch University.

Hibert, F., Taberlet, P., Chave, J., Scotti-Saintagne, C., Sabatier, D., & Richard-Hansen, C. (2013). Unveiling the diet of elusive rainforest herbivores in next generation sequencing era? The tapir as a case study. *PLoS One*, *8*(4), e60799.

Huson, D. H., Mitra, S., Ruscheweyh, H. J., Weber, N., & Schuster, S. C. (2011). Integrative analysis of environmental sequences using MEGAN 4. *Genome Research*, *21*, 1552–1560.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., … Drummond, A. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, *28*, 1647–1649.

Kirkwood, R., & Goldsworthy, S. (2013). *Fur Seals and Sea Lions*. Collingwood, Vic.: CSIRO Publishing.

Ling, J. K. (1992). *Neophoca cinerea*. *Mammalian Species*, *392*, 1–7.

McIntosh, R. R., Page, B., & Goldsworthy, S. D. (2006). Dietary analysis of regurgitates and stomach samples from free-living Australian sea lions. *Wildlife Research*, *33*, 661–669.

McLaughlin, M. J., & Sainani, K. L. (2014). Bonferroni, Holm, and Hochberg corrections: Fun names, serious changes to p values. *PM R*, *6*, 544–546.

Murray, D. C., Bunce, M., Cannell, B. L., Oliver, R., Houston, J., White, N. E., … Haile, J. (2011). DNA-based faecal dietary analysis: A comparison of qPCR and high throughput sequencing approaches. *PLoS One*, *6*(10), e25776.

Murray, D. C., Coghlan, M. L., & Bunce, M. (2015). From benchtop to desktop: Important considerations when designing amplicon sequencing workflows. *PLoS One*, *10*, e0124671.

Oksanen, J., Guillaume Blanchet, F., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., … Wagner, H. (2016). *vegan: Community Ecology Package*.

R package version 2.3-0. Retrieved from http://CRAN.R-project.org/package=vegan, in: Oksanen, J. (Ed.).

Osterrieder, S. K., Salgado Kent, C., & Robinson, R. W. (2015). Variability in haul-out behaviour by male Australian sea lions *Neophoca cinerea* in the Perth metropolitan area, Western Australia. *Endangered Species Research*, *28*, 259–274.

Osterrieder, S. K., Salgado Kent, C., & Robinson, R. W. (2016). Responses of Australian sea lions, *Neophoca cinerea*, to anthropogenic activities in the Perth metropolitan area, Western Australia. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *27*, 414–435.

Pearce, A. F., & Feng, M. (2013). The rise and fall of the "marine heat wave" off Western Australia during the summer of 2010/2011. *Journal of Marine Systems*, *111–112*, 139–156.

Peters, K. J., Ophelkeller, K., Bott, N. J., Deagle, B. E., Jarman, S. N., & Goldsworthy, S. D. (2014). Fine-scale diet of the Australian sea lion (*Neophoca cinerea*) using DNA-based analysis of faeces. *Marine Ecology*, *36*, 347–367.

Peters, K. J., Ophelkeller, K., Bott, N. J., & Goldsworthy, S. D. (2015). PCR-based techniques to determine diet of the Australian sea lion (*Neophoca cinerea*): A comparison with morphological analysis. *Marine Ecology*, *36*, 1428–1439.

Pompanon, F., Deagle, B. E., Symondson, W. O. C., Brown, D. S., Jarman, S. N., & Taberlet, P. (2012). Who is eating what: Diet assessment using next generation sequencing. *Molecular Ecology*, *21*, 1931–1950.

Quemere, E., Hibert, F., Miquel, C., Lhuillier, E., Rasolondraibe, E., Champeau, J., … Chikhi, L. (2013). A DNA metabarcoding study of a primate dietary diversity and plasticity across its entire fragmented range. *PLoS One*, *8*, e58971.

R Core Team (2015). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Radulovici, A. E., Sainte-Marie, B., & Dufresne, F. (2009). DNA barcoding of marine crustaceans from the Estuary and Gulf of St Lawrence: A regional-scale approach. *Molecular Ecology Resources*, *9*, 181–187.

Roberts, D. W. (2016). *labdsv: Ordination and Multivariate Analysis for Ecology*. R package version 1.8-0.

Shehzad, W., McCarthy, T. M., Pompanon, F., Purevjav, L., Coissac, E., Riaz, T., & Taberlet, P. (2012). Prey preference of snow leopard (*Panthera uncia*) in South Gobi, Mongolia. *PLoS One*, *7*(2): e32104.

Shehzad, W., Riaz, T., Nawaz, M. A., Miquel, C., Poillot, C., Shah, S. A., … Taberlet, P. (2012). Carnivore diet analysis based on next-generation sequencing: Application to the leopard cat (*Prionailurus bengalensis*) in Pakistan. *Molecular Ecology*, *21*, 1951–1965.

Taylor, P. G. (1996). Reproducibility of ancient DNA sequences from extinct Pleistocene fauna. *Molecular Biology and Evolution*, *13*, 283–285.

Thomas, A. C., Jarman, S. N., Haman, K. H., Trites, A. W., & Deagle, B. E. (2014). Improving accuracy of DNA diet estimates using food tissue control materials and an evaluation of proxies for digestion bias. *Molecular Ecology*, *23*, 3706–3718.

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APPENDIX

TABLE A1 Sample collection data; details of collection dates and sites and number of scats collected

Reserve	Ocean	No. samples	Date collected
Houtman Abrolhos Nature Reserve	Indian	5	10 April 2013
Beagle Islands Nature Reserve	Indian	8	17 May 2013
Shoalwater Islands Nature Reserve	Indian	1	October 2012
		5	20/21 January 2013
		4	22 May 2013
Fitzgerald River Nature Reserve	Southern	6	October 2012
Recherche Archipelago Nature Reserve	Southern	4	26 January 2013
		1	27 January 2013

TABLE A2 Single source analysis of metabarcoding assays; details of assays tested against DNA extracted from single source samples (barcode size in brackets)

		Metabarcoding assay and % match of query to reference			
Class	Assignment	Crust (170 bp)	Fish (200 bp)	S_Ceph (70 bp)	Ceph (200 bp)
Actinopterygii	*Encrasicholina punctifer*		99–100		
	Hyporhamphus melanochir		99–100		
	Sardinops (*sagax*/*neopilchardus*)		99–100		
	Spratelloide srobustus		99–100		
Malacostraca	*Fenneropenaeus merguiensis*	99–100			
	Portunus pelagicus	99–100			
Cephalopoda	*Nototodarus sloanii*				98–100
	Ommastrephidae (*Martialia hyadesi*/*Nototodarus sloanii*/*Todarodes filippovae*)			97–100	

TABLE A3 Number of samples producing results for each assay; the total number of samples from each site is in brackets

	Houtman Abrolhos (6)	Beagle (8)	Shoalwater Bay (10)	Fitzgerald (6)	Recherche Archipelago (5)
Assay	Number of samples producing results				
Bird 12S	0	3	0	0	0
Ceph 16S	4	6	9	4	1
Crust 16S	3	3	5	0	0
Fish 16S	4	5	8	4	3
Mam 16S	6	8	10	6	5
Plank COI	5	8	10	5	4
S_Ceph 16S	4	6	9	6	5

TABLE A4 Numbers of sequences per assay, per site; "Unfiltered" refers to sequences that have been 100% matched to the sequence specific primers, the MID tags, and the adaptor sequence

Site	Sequence type	Ceph 16S	S_Ceph 16S	Fish 16S	Plank COI	Crust 16S
Houtman Abrolhos	Unfiltered	36225	114540	56420	30530	34167
	Mean unique	395 ± 431	694 ± 453	1487 ± 367	324 ± 138	622 ± 504
	Filtered and assigned	33164	108929	42584	28499	30999
	Mean unique	55 ± 16	95 ± 13	34 ± 25	65 ± 25	34 ± 12
Beagle Islands	Unfiltered	23183	147703	73954	42126	30913
	Mean unique	327 ± 403	555 ± 208	1685 ± 479	309 ± 126	898 ± 518
	Filtered and assigned	19909	140073	53570	38603	25559
	Mean unique	35 ± 14	88 ± 23	25 ± 4	77 ± 32	47 ± 21
Shoalwater Bay	Unfiltered	34613	146541	92175	52095	81945
	Mean unique	331 ± 250	479 ± 331	1385 ± 745	259 ± 148	862 ± 266
	Filtered and assigned	29110	125647	50835	41130	41899
	Mean unique	46 ± 19	81 ± 22	26 ± 18	43 ± 18	51 ± 8
Fitzgerald River	Unfiltered	7754	50363	94898	15122	0
	Mean unique	168 ± 125	368 ± 143	2301 ± 742	198 ± 113	0
	Filtered and assigned	3624	45758	57549	14045	0
	Mean unique	59 ± 47	74 ± 11	37 ± 25	60 ± 40	0
Recherche Archipelago	Unfiltered	332	55926	36321	20501	0
	Mean unique	50	534 ± 124	1239 ± 165	218 ± 160	0
	Filtered and assigned	325	46432	27500	19472	0
	Mean unique	48	62 ± 31	29 ± 13	62 ± 42	0

"Filtered and assigned" refers to the number of sequences that have passed through the Usearch filtering process and were assigned to taxa. "Mean unique" refers to the mean number of unique sequences produced given the number of positive samples for the assay.

TABLE A5 Actinopterygii (ray-finned fishes) identified with Plank COI and Fish 16S assays

Order	Family	Genus/species	Common name	Abrolhos	Beagle	Shoalwater	Fitzgerald	Recherche
Anguilliformes	Congridae	*Conger*	Genus of Conger eels		Plank (1)	Plank (1)	Plank (2), Fish (2)	
		Gnathophis	Genus of Conger eels				Fish (1)	
	Muraenidae		Knot-eels	Fish (1)				Fish (1)
		Gymnothorax pseudothyrsoideus	Highfin Moray	Fish (1)				
Aulopiformes	Aulopidae		Aulopus				Plank (2)	Plank (3)
		Aulopus purpurissatus	Sergeant Baker				Fish (3)	Fish (1)
Beloniformes	Exocoetidae		Flying fishes		Fish (1)			
Beryciformes	Anoplogastridae		Fangtooths				Fish (1)	
	Berycidae	*Centroberyx australis*	Yellow-eyed Red Snapper					Fish (1)
		Centroberyx gerrardi	Red Snapper					Plank (2)
Clupeiformes	Clupeidae		Herrings		Fish (1)			
		Etrumeus	Maray				Plank (1)	
		Sardinops sagax	Australian Sardine				Fish (1)	
	Engraulidae	*Engraulis*	Anchovies		Fish (1)			
Perciformes			Perch-like Fishes			Fish (1)		
	Labridae		Wrasses		Fish (1)			
		Coris auricularis	Western King Wrasse		Plank (1)			
		Leptoscarus vaigiensis	Marbled Parrotfish	Fish (1)				
		Notolabrus	Wrasses		Plank (2)			
		Odax acroptilus	Marine Rainbowfish		Fish (1)			
		Odax cyanomelas	Herring Cale				Plank(2), Fish (1)	
	Mullidae	*Parupeneus*	Common Goatfish	Fish (1)				
		Parupeneus spilurus	Blacksaddle Goatfish	Plank (1)				
		Upeneichthys	Goatfish		Fish (1)	Fish (1)	Fish (1)	
		Upeneichthys stotti	Stott's Goatfish		Fish (1)		Fish (1)	
	Pempheridae	*Parapriacanthus*	Bullseyes				Fish (1)	
	Pomacanthidae	*Pomacanthus semicirculatus*	Blue Angelfish	Fish (1)				
	Pomacentridae		Damselfishes	Plank (2)	Plank (1)			

(Continues)

TABLE A5 (Continued)

Order	Family	Genus/species	Common name	Abrolhos	Beagle	Shoalwater	Fitzgerald	Recherche
		Chromis	Damselfishes		Fish (1)			
		Parma microlepis	White Ear		Fish (1)			
	Siganidae	*Siganus*	Rabbitfish		Fish (2), Plank (2)	Fish (1)		
Scorpaeniformes			Scorpion Fishes & Sculpins				Fish (1)	
	Platycephalidae	*Leviprora inops*	Crocodile Flathead	Plank (2)	Plank (1)	Plank (4)		
		Platycephalus aurimaculatus	Tiger Flathead				Plank (2)	
		Platycephalus longispinis	Long-spine Flathead				Plank (1)	
	Scorpaenidae	*Scorpaenodes*	Scorpion Fish				Fish (1)	
Siluriformes	Plotosidae		Blunt-tail Catfishes		Plank (1)			
Tetraodontiformes	Monacanthidae		Leatherjackets		Fish (1)			
		Chaetodermis penicilligera	Tasselled Leatherjacket		Fish (1), Plank (1)	Fish (2), Plank (1)		
		Eubalichthys mosaicus	Mosaic Leatherjacket		Fish (1)			
		Monacanthus chinensis	Fanbelly Leatherjacket		Fish (1)			
		Nelusetta ayraudi	Ocean Jacket				Plank (1)	
		Scobinichthys granulatus	Rough Leatherjacket		Fish (1), Plank (2)			
		Thamnaconus	Leatherjacket			Fish (1)		
	Tetraodontidae	*Omegophora*	Toadfish				Fish (1)	
		Omegophora armilla	Ringed Toadfish				Plank (2)	
Zeiformes	Zeidae	*Zeus faber*	John Dory					Fish (1)

The number of samples in which the taxa were detected is indicated in the brackets.

TABLE A6 Chondritchthyes (sharks and rays) identified with Plank COI and Fish 16S assays

Order	Family	Genus/species	Common name	Abrolhos	Beagle	Shoalwater	Fitzgerald	Recherche
Carcharhiniformes			Ground sharks			Fish (2)		Plank (1)
	Scyliorhinidae	*Aulohalaelurus labiosus*	Black Spotted Catshark		Plank (1)	Plank (6)		
	Triakidae	*Mustelus*	Gummy shark					Fish (1), Plank (1)
Heterodontiformes	Heterodontidae	*Heterodontus*				Fish (1)		
		Heterodontus portusjacksoni	Port Jackson Shark		Plank (1)	Plank (2)		
Myliobatiformes	Myliobatidae	*Myliobatis australis*	Southern Eagle Ray			Plank (1)		
	Urolophidae		Stingarees/Round Rays			Fish (1)		
		Trygonoptera				Plank (1)	Plank (1)	
		Trygonoptera mucosa	Western Shovelnosed Stingaree				Plank (1)	
		Trygonoptera personata	Masked Stingaree			Plank (3)	Plank (1)	
		Urolophus lobatus	Lobed Stingaree			Plank (3)	Plank (1)	
		Urolophus paucimaculatus	Sparsely Spotted Stigaree			Plank (1)	Plank (1)	
Orectolobiformes	Orectolobidae		Wobbegongs	Plank (4)		Plank (3)		
		Orectolobus		Fish (2)		Fish (2)		
Rhinobatiformes	*Rhinobatidae*		Guitar fishes	Fish (1)		Fish (2)		
		Aptychotrema vincentiana	Western Shovelnose Ray	Plank (1)		Plank (1)		
		Trygonorrhina guanerius	Southern Fiddler Ray			Fish (1)		

The number of samples in which the taxa was detected is indicated in the brackets.

TABLE A7 Cephalopod and Gastropod taxa identified with Ceph 16S, S_Ceph 16S, and Plank COI assays

Class	Order	Family	Genus/species	Common name	Abrolhos	Beagle	Shoalwater	Fitzgerald	Recherche
Cephalopoda	Octopodiformes	Octopodidae		Octopus	S_Ceph (2)	S_Ceph (8)	S_Ceph (1)	S_Ceph (2) Ceph (1)	
			*Grimpella thaumastocheir**	Velvet Octopus	S_Ceph (1)	S_Ceph (2)			
			Octopus		S_Ceph (4), Plank (2), Ceph (1)	S_Ceph (6), Plank (3)	S_Ceph (5), Plank (4)	S_Ceph (2)	S_Ceph (2)
			*Octopus ornatus**		Ceph (1)	Ceph (1)			
			Octopus tetricus	Gloomy Octopus	S_Ceph (1)	S_Ceph (3)	S_Ceph (5)		
			*Octopus vulgaris**	Common Octopus		S_Ceph (3), Ceph (4)	S_Ceph (4), Ceph (5)	Ceph (1)	
			Scaeurgus					S_Ceph (1)	
	Oegopsida	Ommastrephidae	*Nototodarus gouldi/ Todarodes pacificus**						S_Ceph (2)
			Nototodarus gouldi	Red Arrow Squid					Ceph (1)
	Myopsida	Loliginidae	*Sepioteuthis australis*	Southern Calamari Squid	S_Ceph (1), Ceph (1)	S_Ceph (1), Ceph (1)		S_Ceph (2), Ceph (1)	S_Ceph (2)
	Sepiida	Sepiidae		Cuttlefish					S_Ceph (1), Plank(1)
			Sepia apama	Giant Cuttlefish	S_Ceph(4), Plank (4), Ceph (4)	S_Ceph (5), Plank (3), Ceph (5)	S_Ceph (8), Plank (5), Ceph (7)	S_Ceph (5), Plank (4), Ceph (1)	S_Ceph (2)
		Sepiolidae		Dumpling Squids				S_Ceph (1)	
Gastropoda		Haliotidae	*Haliotis*			S_Ceph (1)	S_Ceph (1), Ceph (1)		
			Haliotis diversicolor	Many Colored Abalone		S_Ceph (2)			
		Trochidae	*Stomatella impertusa*	False ear shell				S_Ceph (1)	

The number of samples in which the taxa were detected is indicated in the brackets. Those species found in Australia, but not in the area of collection, are indicated by an asterisk (*).

TABLE A8 Crustaceans and Aves detected using Crust 16S, Bird 12S, and Plank COI assays

Class	Order	Family	Genus/species	Common name	Abrolhos	Beagle	Shoalwater	Fitzgerald	Recherche
Malacostraca	Decapoda			Crustaceans			Crust (1)		
		Palinuridae	*Panulirus cygnus*	Western Rock Lobster	Crust (1)	Crust (3)	Crust (2)		
		Portunoidea	*Thalamita danae*	Unknown Swimmer Crab	Crust (1)				
		Xanthidae		Crabes de Boue	Crust (1)				
Aves	Charadriformes	Laridae	*Onychoprion anaethetus*	Bridled Tern		Plank (1)			
	Suliformes	Phalacrocoracidae	*Phalacrocorax varius*	Pied Cormorant		Plank (2), Bird (3)			

The number of samples in which the taxa were detected is indicated in the brackets.

TABLE A9 Fish 16S OTU sequence abundance per site and per ocean

OTU ID	Abrolhos	Beagle	Shoalwater	Fitzgerald	Recherche	Indian Ocean	Southern Ocean
OTU_1	13,242	0	0	0	0	13,242	0
OTU_7	7,980	0	0	0	0	7,980	0
OTU_16	8,964	0	0	0	0	8,964	0
OTU_29	1,532	0	0	0	0	1,532	0
OTU_30	1,441	0	0	0	0	1,441	0
OTU_34	1,536	0	0	0	0	1,536	0
OTU_38	267	0	0	0	0	267	0
OTU_6	0	8,456	0	0	0	8,456	0
OTU_12	0	7,816	0	0	0	7,816	0
OTU_19	0	4,092	0	0	0	4,092	0
OTU_21	0	3,106	0	0	0	3,106	0
OTU_27	0	2,165	0	0	0	2,165	0
OTU_28	0	1,685	0	0	0	1,685	0
OTU_31	0	1,160	0	0	0	1,160	0
OTU_35	0	851	0	0	0	851	0
OTU_8	0	11,384	3,620	0	0	15,004	0
OTU_10	0	8,972	10,141	0	0	19,113	0
OTU_14	0	0	5,014	0	0	5,014	0
OTU_15	0	0	7,118	0	0	7,118	0
OTU_17	0	0	4,395	0	0	4,395	0
OTU_20	0	0	3,137	0	0	3,137	0
OTU_22	0	0	2,719	0	0	2,719	0
OTU_23	0	0	5,008	4	0	5,008	4
OTU_25	0	0	2,386	0	0	2,386	0
OTU_11	4,329	0	6,858	96	0	11,187	96
OTU_5	0	46	10,736	10,787	0	10,782	10,787
OTU_36	0	117	0	1,659	0	117	1,659
OTU_37	0	17	0	315	0	17	315
OTU_3	0	0	0	11,830	0	0	11,830
OTU_4	0	0	0	11,695	0	0	11,695
OTU_13	0	0	0	7,989	0	0	7,989
OTU_24	0	0	0	2,414	0	0	2,414
OTU_32	0	0	0	1,913	0	0	1,913
OTU_2	0	0	0	16,951	10,632	0	27,583
OTU_9	0	0	0	0	7,835	0	7,835
OTU_18	0	0	0	0	4,375	0	4,375
OTU_26	0	0	0	0	2,016	0	2,016
OTU_33	0	0	0	0	1,011	0	1,011

PLOS GENETICS

RESEARCH ARTICLE

Marine environmental DNA biomonitoring reveals seasonal patterns in biodiversity and identifies ecosystem responses to anomalous climatic events

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Abstract

Marine ecosystems are changing rapidly as the oceans warm and become more acidic. The physical factors and the changes to ocean chemistry that they drive can all be measured with great precision. Changes in the biological composition of communities in different ocean regions are far more challenging to measure because most biological monitoring methods focus on a limited taxonomic or size range. Environmental DNA (eDNA) analysis has the potential to solve this problem in biological oceanography, as it is capable of identifying a huge phylogenetic range of organisms to species level. Here we develop and apply a novel multi-gene molecular toolkit to eDNA isolated from bulk plankton samples collected over a five-year period from a single site. This temporal scale and level of detail is unprecedented in eDNA studies. We identified consistent seasonal assemblages of zooplankton species, which demonstrates the ability of our toolkit to audit community composition. We were also able to detect clear departures from the regular seasonal patterns that occurred during an extreme marine heatwave. The integration of eDNA analyses with existing biotic and abiotic surveys delivers a powerful new long-term approach to monitoring the health of our world's oceans in the context of a rapidly changing climate.

Author summary

All environments contain genetic remnants of the life they contain and support. For example, samples collected from the ocean contain biological material such as microscopic organisms, shed cells, excrement and saliva—the DNA from which reveals the

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surrounding marine biodiversity. Environmental DNA (eDNA) approaches have the ability to identify marine species that are notoriously difficult to identify using morphology alone. Here we develop, and apply, a DNA 'toolkit' to five years of samples collected from a single site in the Indian ocean. It is rare to find a temporal series of samples of this duration that are also suitable for DNA analysis. We show that eDNA techniques have the capacity to monitor ocean biology in fine detail. We demonstrate how the biological communities of plankton and fish respond to normal seasonal changes and, more importantly, to that of an uncharacteristic heatwave. The methods embodied in this paper are applicable to marine studies across the globe and, as such, pave the way for the design of long-term monitoring programs using eDNA.

Introduction

Changes in ocean temperatures, chemistry and currents are occurring faster now than at any time in human history [1, 2]. These changes will certainly impact the productivity in marine environments that is critical for social and economic wellbeing [3]. These impacts have driven the expansion of global efforts to monitor marine biota and track ecosystem health [1, 4, 5]. Abiotic environmental data are already collected by various methods across all oceans [4, 6], but thorough sampling of marine biota is far more restricted and challenging [5]. Robust biomonitoring programs that link biological changes to the physio-chemical state of the oceans will help to identify ecological trends and predicting future trajectories.

Since 1931, the biomass and morphological species in zooplankton communities have been used extensively for oceanic biomonitoring [7]. Zooplankton are the trophic link between phytoplankton and larger predators [8]. These highly diverse communities have been described as 'beacons of change' [9], as their community composition is known to respond to fluctuations in both abiotic and biotic factors [5, 9, 10]. Most zooplankton are ectothermic, so they are sensitive to temperature changes that affect their physical activity and physiology [9]. Many species are also fast growing and short-lived and so communities typically respond rapidly to changes in environmental conditions [5, 9–11].

The importance of extended temporal sampling to describe changes within planktonic communities has long been recognised [1, 4, 5, 12–14]. A long-term analysis has the ability to define baselines and understand what is 'normal' for a community [4] and provides a mechanism to gauge ecosystem health [11]. There are several extended studies targeting zooplankton [1, 4, 5, 12, 14–17], yet these typically focus on a narrow range of taxa [1, 11, 13, 18–20].

Morphological identification of zooplankton is time consuming and expensive [4, 21]. Samples must be in good physical condition, particularly for taxonomic identifications reliant on the presence of fragile appendages. This problem is worst for easily damaged, soft-bodied phyla such as Cnidaria and Ctenophora [22]. Many marine animals, including fish and larger crustaceans, have a larval planktonic phase, and identification of larvae to species is difficult or impossible, even for skilled taxonomists [21, 23, 24]. Morphological studies tend to overestimate the relative abundance of those taxa that are readily identified, but overlook a significant fraction of marine animal groups. Consequently there is growing recognition that morphology by itself will struggle to meet with the increasing need for holistic marine biomonitoring in conservation and management decisions [4, 25].

Environmental DNA (eDNA) is transforming our ability to study marine biodiversity. Recent metabarcoding studies on eDNA extracted from water [26–28], sediment [29], scat [25, 30–33] and plankton [21, 34] demonstrate its capacity to profile a vast range of biota. While

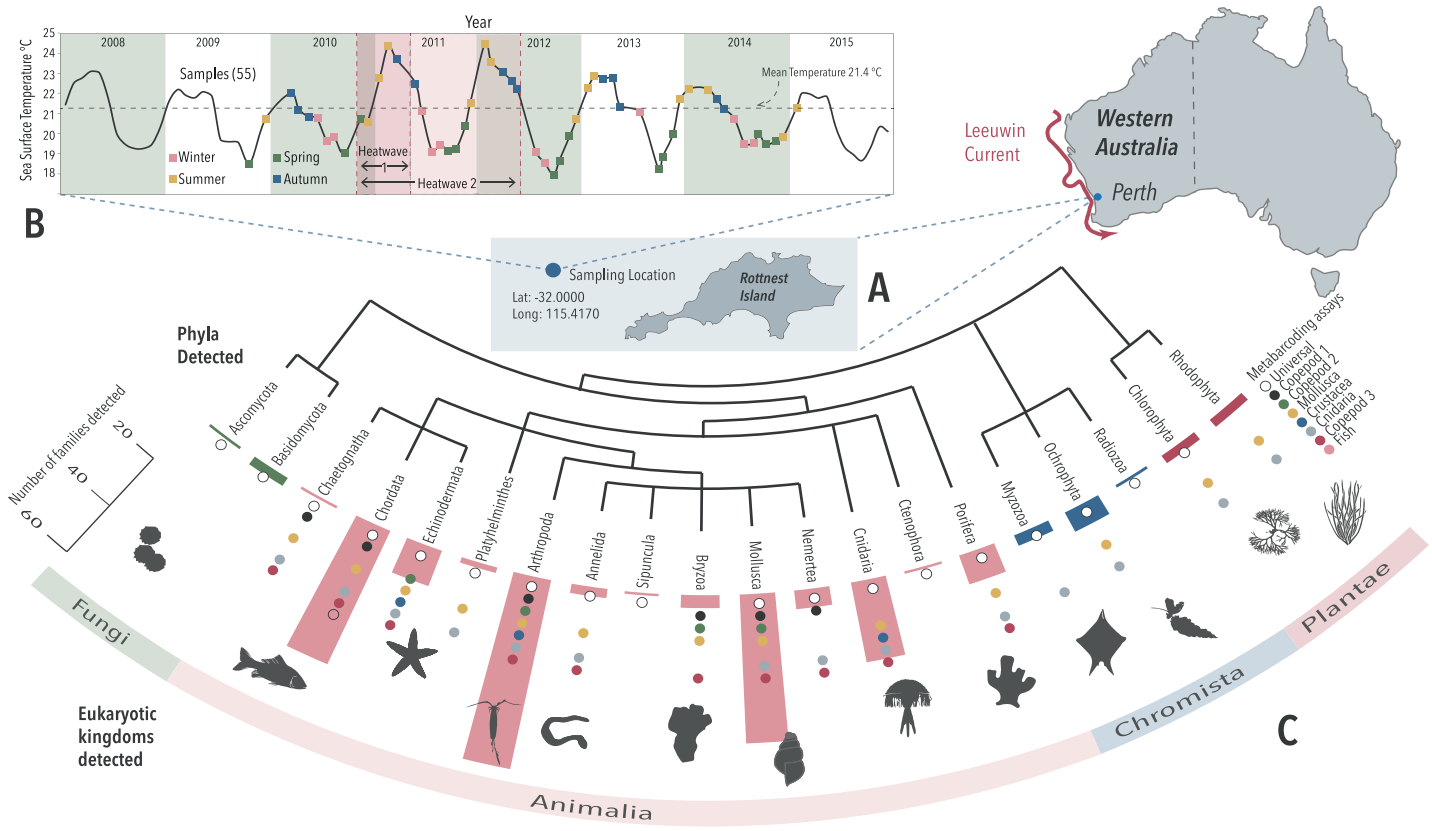

Fig 1. Extent of marine taxa revealed by eDNA from Rottnest Island (A). 55 monthly plankton samples taken across five years (2009–2015) and an extreme heatwave event (B), which yielded 245 families of eukaryotic zooplankton across 20 phyla (C).

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these studies focus strongly on spatial and community differences, the ability for eDNA to act as a long-term temporal biomonitoring tool is unexplored.

Environmental DNA is defined in Taberlet *et al* [35] as a "*complex mixture of genomic DNA from many different organisms found in an environmental sample. . . [including] material resulting from filtering air or water, from sifting sediments, or from bulk samples*". Here, due to historic sampling, we analyse eDNA purified from bulk zooplankton samples systematically collected monthly over five-years from a single ecologically significant site at Rottnest Island, Western Australia [36] (Fig 1A). This temporal window of sampling includes a "marine heatwave" anomaly that had significant impacts on the south Western Australian marine ecosystem [37–39]. We test the capacity for eDNA metabarcoding to track biotic shifts, examine how eDNA signatures relate to abiotic variables, and lastly outline the value and practical implementation of multi-year eDNA programs

Shotgun DNA sequencing has been used for eDNA community analysis [40] but it is cost prohibitive and dominated by prokaryotic taxa [28]. Single marker metabarcoding approaches have proven useful for biological monitoring, but their taxonomic focus has to be narrow because each assay is by definition limited in scope. Even supposedly "universal" DNA metabarcoding assays have proven inadequate to identify a comprehensive range of target taxa in our global oceans [28]. To address the challenge of pinpointing a range of metazoan taxa, we

developed a novel multi-gene (COI, 16S & 18S) metabarcoding 'toolkit' capable of working with both degraded and intact eDNA, and able to identify a wide variety of taxa found within zooplankton communities. We used three existing metabarcoding assays and designed five more (S1 Table) to target a range of crustaceans, molluscs, fish and cnidarians known to be present at the reference site [41]—a site that has been monitored using a variety of methods since 1951 [6].

Results and discussion

Overall, while the majority of the eDNA extracted during this study originates from the plankton sampled (including larva and eggs), a small amount (impossible to quantify) would derive from sloughed cells or faecal material from larger organisms. From this total DNA more than four hundred distinct eukaryotic taxa were identified in this five-year study. These taxa were identified from more than nine million metabarcode sequences clustered into four thousand unique high abundance groups. Across all time points and assays, a total of 20 eukaryotic phyla were detected containing 245 families (Fig 1; S2–S7 Tables). Fig 1B also depicts the surface temperature and chronology of collection at the monitoring site. Most detections (70%) were within Arthropoda (including 62 families) and, of these, 87% were from Hexanauplia (including 24 families), the class that contains all copepods. The metabarcoding method employed here identified some of the gelatinous and larval zooplankton such as over 15 genera of hydrozoa and 50 genera of actinopterygii, many to species level. In practice, all assays, with the exception of the Fish assay, detected an extremely broad range of taxa. The Copepod 3 assay alone was responsible for over 1100 assignments across ten Animalia phyla; almost a quarter of all detections. It is, however, the integration of all assays that has revealed some of the breadth of biodiversity within this ecosystem over the five-year period. Had the study been limited to the 18S Universal assay, fewer than 70 assignments would have been made.

While Fig 1C showcases the taxa that our assays detected, more than 40% of the DNA sequences could not be reasonably assigned within a taxonomic framework. As a consequence of this problem, we applied a taxonomy-independent approach so that the analyses were not biased by the limitations of reference databases or the accuracy of the underpinning taxonomy. Operational Taxonomic Units (OTUs) enabled a more comprehensive exploration of the correlations between biotic and abiotic change over time.

Seasonal & annual patterns

Biological monitoring at a single point in time is typically inadequate to describe total biodiversity or to explore changes in diversity over time. Collecting multiple time-stamped samples reveals greater total (gamma) biodiversity and allows measurement of beta diversity as a temporal change. For each assay, OTU biodiversity analysis involved both counting of the number of discrete OTUs—a measure hereafter referred to as "Richness"—and the presence/absence composition of the OTUs—referred to as "Assemblage". OTUs from each assay were examined independently so that comparisons were all made within the same experimental frameworks.

There are varying approaches for presenting eDNA metabarcoding data in terms of Assemblage and Richness. Some authors rarefy their data to normalise results for differing sequencing depth among libraries. We made the decision not to do this because sequence number and OTU accumulation curves had plateaued for each sample indicating that we had sampled the majority of the OTUs in each case (For example; S1 Fig), Pearson's correlation tests showed there was no evidence to suggest a significant correlation between the number of sequences (i.e. sequencing depth) and the number of OTUs obtained for the 18S and 16S assays (S2 Fig). However, sequencing depth and number of OTUs (Richness) were moderately correlated

Table 1. Significance of changes to the Operational Taxonomic Unit (OTU) Richness (a count of the number of OTUs in each sample) & Assemblage (the OTUs making up each sample) during different time periods within the five-year eDNA data including F statistics (F)—PERMANOVA+ [42].

Assay (Number of individual OTUs)	OTU diversity test	Main tests 2010–2014			Main tests Before, During and After	
		Month df (30,51)	Season df (15,51)	Year df (4,51)	Heatwave 1 Nov 2010 –April 2011; df (2,54)	Heatwave 2 Nov 2010 –May2012; df (2,54)
Cnidaria	Richness	- F = 2.03	- F = 0.93	- F = 1.16	- F = 1.03	- F = 0.16
(246 OTUs)	Assemblage	- F = 0.91	** F = 1.38	** F = 1.58	*** F = 2.80	*** F = 2.84
Copepod 1	Richness	- F = 4.89	- F = 1.21	** F = 4.63	** F = 6.60	* F = 4.16
(171 OTUs)	Assemblage	* F = 2.15	* F = 1.41	*** F = 1.99	*** F = 3.28	*** F = 3.64
Copepod 2	Richness	- F = 2.76	- F = 0.71	- F = 1.50	- F = 1.67	- F = 0.34
(124 OTUs)	Assemblage	- F = 0.10	- F = 0.22	- F = 1.48	- F = 1.48	* F = 2.01
Copepod 3	Richness	- F = 2.76	- F = 0.71	- F = 1.50	- F = 1.67	- F = 0.34
(342 OTUs)	Assemblage	**F = 2.31	*** F = 1.48	- F = 1.35	**F = 1.94	**F = 2.28
Crustacea	Richness	- F = 0.55	- F = 1.26	- F = 1.96	- F = 2.41	* F = 3.59
(132 OTUs)	Assemblage	- F = 0.86	* F = 1.29	- F = 1.15	- F = 1.08	- F = 1.32
Fish	Richness	- F = 2.38	** F = 3.49	- F = 0.58	- F = 0.09	- F = 0.21
(87 OTUs)	Assemblage	*F = 2.88	*** F = 1.77	- F = 0.79	- F = 1.18	- F = 0.85
Mollusca	Richness	- F = 2.21	- F = 1.27	- F = 0.32	- F = 0.57	- F = 0.47
(345 OTUs)	Assemblage	- F = 1.00	*** F = 1.65	* F = 1.39	*** F = 2.41	*** F = 2.29
Universal	Richness	- F = 0.52	- F = 0.96	- F = 2.02	- F = 0.40	- F = 1.78
(97 OTUs)	Assemblage	- F = 0.96	* F = 1.34	- F = 1.45	** F = 2.08	*** F = 2.45

Where ***is $P \leq 0.001$, **is $P \leq 0.01$, *is $P \leq 0.05$ & – is no significant change

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(R^2<0.522) for the COI assays (S2 Fig). Nevertheless, as sequencing depth variation is spread evenly across the samples (S2 Fig), we consider it unlikely that Richness or Assemblage estimates are compromised by this data treatment.

Our initial analyses of eDNA (Table 1) demonstrated strong seasonality in the Assemblage from those assays that predominately detect meroplankton, including fish, molluscs and cnidarians. This seasonality was not reflected in Richness, with the exception of the Fish assay. A pairwise analysis between seasons (S8 Table) indicated that the most consistent differences in Assemblage were detected between summer:winter, followed by spring:winter and spring: autumn. The least significant Assemblage changes were identified by the assays that predominantly detect holoplankton e.g. the Copepod assays. These detected no significant changes (after *post-hoc* correction) between winter:autumn, and summer:spring. These results provide a detailed example for multi-year marine biodiversity surveys based on eDNA.

The Fish assay revealed strong seasonality in both Richness and Assemblage (Fig 2). A pairwise analysis showed significant changes between all seasons for the Assemblage as well as Richness (S8 Table), the two exceptions were for Richness between the adjacent seasons summer:spring and winter:autumn. Most fish are only present in the zooplankton community after broadcast spawning their eggs or during their pelagic larval phase, so these seasonal changes make biological sense [24]. Seasonal fluctuations have been previously observed in fish using eDNA extracted from water [43, 44]. However, these studies were limited to durations of six and twelve months respectively. The current study provides additional and enduring evidence for the ability of eDNA to detect of seasonality over an extended period (5 years) and further incorporates a much broader range of biodiversity.

OTUs that characterise particular time periods were identified by *indval* analysis [45]. The strong seasonality in the Fish OTUs suggests that they might be driving significant differences

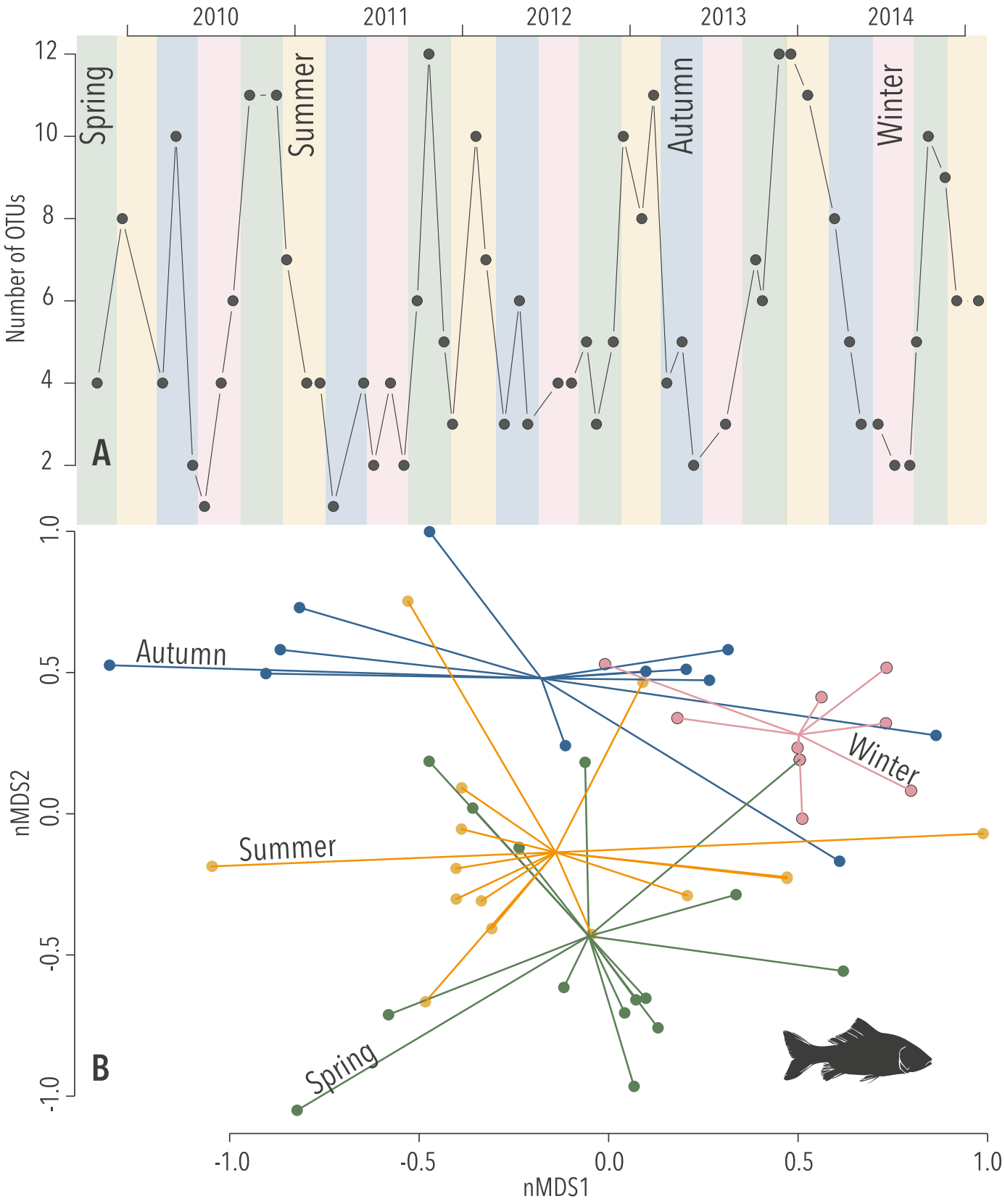
2010
2011
2012
2013
2014
Spring
Summer
Autumn
Winter
Number of OTUs
12
10
8
6
4
2
A
1.0
0.5
0.0
-0.5
-1.0
nMDS2
Autumn
Winter
Summer
Spring
B
-1.0
-0.5
0.0
0.5
1.0
nMDS1

Fig 2. Seasonality in eDNA revealed by the Fish assay: (A) Number of Operational Taxonomic Units (OTUs) at each time point (Richness; $p < 0.001$) and (B) Diversity of OTUs as exhibited by a non-parametric multivariate analysis (Assemblage; $k = 3$, stress = 0.15, $p < 0.001$), the coloured lines extrude from the centroids of each season towards the variation of Assemblage in each sample.

https://doi.org/10.1371/journal.pgen.1007943.g002

identified in the seasonal *indval* analyses across all assays (S9 Table), but this was not the case. Spring was characterised by a significant indicator matched to Labridae (a speciose fish family), but *Calcinus dapsiles* (a hermit crab) and *Evadne spinifera* (a water flea) were the summer's four top indicators. *Calcinus dapsiles* are only planktonic as larvae and only present seasonally, but *E. spinifera* is part of the plankton for its entire life.

Flaccisagitta enflata (a chaetognath or predatory arrow worm) and the copepods *Farranula gibbula* and *Centropages orsinii* were the most significant indicators for autumn. The copepods, *Canthocalanus pauper* and *Centropages furcatus* were found in winter. The genetic assignment of *C. orsinii* and *C. furcatus* are of interest as they are typically tropical species found in the Indian Ocean [46] indicating that they are likely to have been swept south by the warm water Leeuwin current (Fig 1A) in each year [47]. These indicator species analyses generate lists of target taxa that provide a more refined picture of seasonal changes in biodiversity—S9 Table lists all significant seasonally variable OTUs.

The years 2010 to 2014 showed changes in the Assemblage identified by several of the assays (Table 1); the pairwise analysis (S10 Table) identified when these changes occurred. The OTUs that most strongly characterise each year are presented in S11 Table. Six assays showed significant changes in Assemblage between 2010 and 2011 and each of the three subsequent years (S10 Table). In particular, the Assemblage from Copepod 1, Mollusca and Cnidaria assays responded strongly. This pattern suggests a biotic regime shift in response to an environmental anomaly. S11 Table lists all significant yearly variable OTUs.

Biotic heatwave effects

The Rottnest Island area has global significance as it is situated within a site of high biodiversity that is largely endemic [36]. This sample set was particularly significant because it encompasses two uncharacteristic summer temperature extremes in 2011 and 2012. The WA marine heatwave was originally defined as occurring between November 2010 and April 2011 [38]. However, similarly high sea surface temperatures (SST) were recorded during the following year [48–50] (Fig 2B & S3 Fig). In this study, periods for the heatwaves were: "Heatwave 1", a five-month heatwave, as described in Pearce and Feng (2013); and "Heatwave 2", which encompasses Heatwave 1 and extends across a 17-month period from November 2010 –May 2012 (Fig 1B). The Assemblage from most assays (except Crustacea, Fish) responded significantly to the designated heatwave periods (Table 1).

The most significant changes in the Assemblage were between the periods pre- and post-Heatwave 1 (S12 Table). For Heatwave 2, significant differences were seen before, after, as well as during the thermal event (S12 Table). Analyses of both heatwave periods suggest that there were significant, and potentially persistent, changes that occurred within the zooplankton communities as a result of these collective temperature anomalies. Only ongoing research will determine whether these changes are permanent, however, climate-mediated change has already been reported in the same study area where Wernberg (et al.) [39] reported that a kelp dominated nearshore ecosystem shifted to a more tropicalised system containing seaweed turf.

The value of employing assays with different taxonomic specificities is shown by the lack of significant heatwave-induced Assemblage changes observed for some assays. No change was detected using the Crustacea and Fish assays. The taxa detected by these assays are generally long-lived with pelagic larval phases, so any significant change in these groups is likely to

occur gradually and would only be detected with an even longer-term study. The Heatwaves had less significant effects on Richness, however the Copepod 1 and 3 assays demonstrated changes in Richness, particularly between before and after the thermal anomaly periods (S12 Table).

The Copepod 1 assay illustrates the effects of Heatwaves 1 and 2 on the Assemblage and Richness (Fig 3). The Copepod 1 assay was designed *in silico* to focus on the genus *Triconia*, but, as is common in metabarcoding approaches, *in vitro*, the assay detects a much wider range of copepods as well as other arthropods.

OTUs characterising the periods defined by the heatwaves were identified by *indval* analysis. The OTUs corresponding to *Paracalanus indicus* (a copepod) and Pythiales (an order of water mould) are strong indicators for the 'before' periods (S13 & S14 Tables). The Copepod 1 OTUs characterising the heatwave 'during' periods were significantly different; only ten OTUs (11%) overlap. The best indicator for Heatwave 1 was Hexanauplia (the class which contains all copepods); this OTU is also an indicator for Heatwave 2 (S13 & S14 Tables). For the 'after' periods, nine OTUs are shared between them (15%). Nine anonymous copepod OTUs (15%) were strongly associated with the 'after' of both heatwave periods. This demonstrates the advantage of the OTU approach and provides an opportunity for taxonomists to link these sequences to the species that they provisionally represent.

These time-stamped metabarcoding data show, for the first time, that eDNA metabarcoding is able to track biotic shifts in response to seasonal and annual changes, as well as identify a known temperature anomaly that threatened global biodiversity hotspots on the west coast of Australia. This result has obvious implications for biomonitoring of oceans in the face of anthropogenic pressures including climate change, acidification, pollution, fishing and aquaculture impacts. The Assemblage and Richness data provided by eDNA metabarcoding can be integrated with other abiotic factors to develop a more holistic picture of how biomes respond to a variety of environmental factors.

Biological response to abiotic change

Biological samples analysed in this study were collected alongside complementary measurements of physical and chemical characteristics of the sampling site. Sea surface temperature (SST) and the concentrations of salinity and silicate (an important nutrient in oceans), were all important explanatory abiotic variables for both Richness and Assemblage across the majority of metabarcoding assays (Table 2). These variables feature in either the 'best' or the most parsimonious alternative models for all of the assays used (Tables 2 and S15).

SST and salinity explained a large portion of the biological variation we observed. The assay most sensitive to the abiotic factors was Copepod 3; where SST, and concentrations of salinity, and silicate explained 22.7% of the variation in Assemblage, and SST and salinity concentration explained 39.2% of the variation in Richness (Table 2).

Richness increased significantly with warmer SST for most assays, with the exception of Copepod 1 and Copepod 2, which showed an insignificant negative relationship to SST (Table 2). Richness conversely decreased with increasing salinity for the Copepod, Crustacean, and Universal assays, but reacted positively in the Cnidaria, Fish, and Mollusca results. Silicate correlations had the opposite pattern, being positively correlated with Richness in the Copepod, Crustacean, and Universal results, but negatively correlated to Richness when measured against the Cnidaria, Fish, and Mollusca assays (Table 2). These results are likely due to an indirect link between the environmental variables to the zooplankton composition via direct links upon the phytoplankton [51]. These results illustrate the different niches that

zooplankton can exploit within an ecosystem. As one group of zooplankton find conditions uninhabitable and diminishes locally, another group will thrive within the niche.

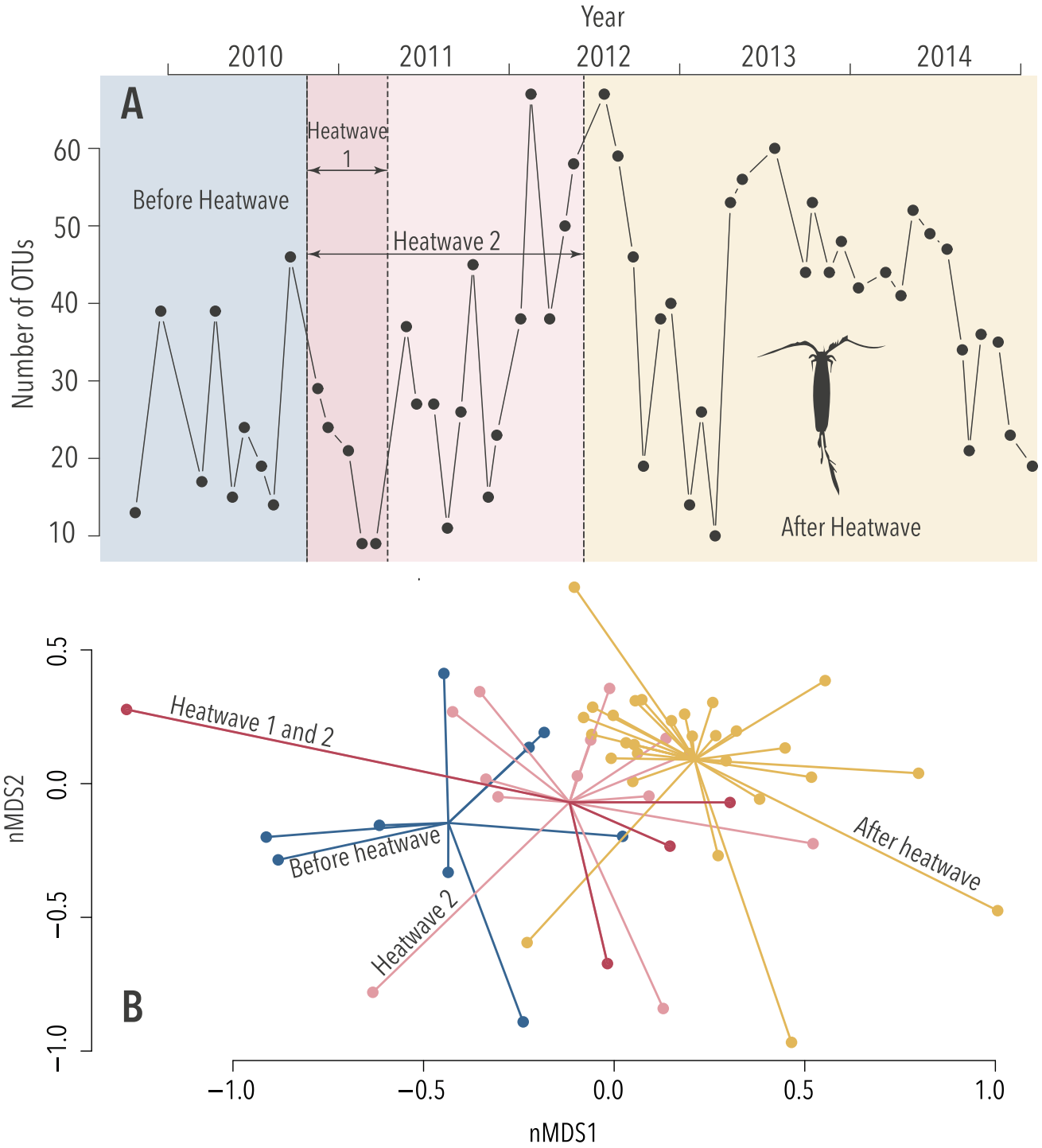

Fig 3. Heatwave effects revealed by Copepod 1 eDNA assay "Heatwave 1" and "Heatwave 2" are indicated. (A) Changes in the number of Operational Taxonomic Units (OTUs) over time (Richness: Heatwave 1; $p < 0.01$ & Heatwave 2; $p < 0.05$) and (B) changes in the diversity of the OTUs revealed by non-parametric multivariate analysis (Assemblage; $k = 3$, stress = 0.15, both $p < 0.001$), the coloured lines extrude from the centroids of each time period towards the variation in Assemblage from each sample.

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Table 2. Relationship between sea surface temperature (SST) and abiotic factors, and OTU richness (the number of OTUs in each sample)—nbGLM (negative binominal Generalised Linear Model; [52])—and assemblage (what OTUs are in each sample)—DistLM (Distance based Linear Model; [42])—as indicated by each assay.

Assay used	OTU diversity test	Variable	SST	Salinity	Silicate	Nitrate	Phosphate	Ammonium	Best Model	
Cnidaria	Assemblage	P	**<0.001**	**<0.001**	**<0.001**	0.072	0.114	0.001	R^2	0.162
		R^2	**0.053**	**0.067**	**0.072**	0.028	0.026	0.044		
	Richness	P	**0.069**	0.061	**0.029**	0.842	0.809	0.700	R^2	0.112
		R^2	**0.056 (+)**	0.059 (+)	**0.078 (-)**	< 0.001 (-)	0.001 (+)	0.002 (-)		
Copepod 1	Assemblage	P	**0.030**	**0.002**	**< 0.001**	0.097	0.020	0.100	R^2	0.155
		R^2	**0.034**	**0.050**	**0.079**	0.028	0.036	0.028		
	Richness	P	0.953	**0.045**	0.308	0.478	0.376	0.245	R^2	0.067
		R^2	< 0.001 (-)	**0.067 (-)**	0.018 (+)	0.009 (+)	0.014 (+)	0.024 (+)		
Copepod 2	Assemblage	P	**0.021**	**< 0.001**	**< 0.001**	0.141	0.264	0.008	R^2	0.230
		R^2	**0.011**	**0.126**	**0.120**	0.027	0.022	0.049		
	Richness	P	0.428	**< 0.001**	**< 0.001**	0.146	0.172	0.011	R^2	0.309
		R^2	0.004 (-)	**0.255 (-)**	**0.204 (+)**	0.036 (+)	0.032 (+)	0.102 (+)		
Copepod 3	Assemblage	P	**0.011**	**<0.001**	**<0.001**	0.043	0.447	0.002	R^2	0.227
		R^2	**0.042**	**0.138**	**0.092**	0.034	0.018	0.053		
	Richness	P	**0.537**	**<0.0001**	0.007	0.252	0.561	**0.045**	R^2	0.392
		R^2	**0.007 (+)**	**0.305 (-)**	0.115 (+)	0.023 (+)	0.006 (+)	**0.067 (+)**		
Crustacea	Assemblage	P	**0.001**	**<0.001**	0.005	0.337	0.479	0.009	R^2	0.098
		R^2	**0.046**	**0.056**	0.038	0.021	0.019	0.037		
	Richness	P	**0.079**	0.246	0.799	0.104	0.629	**0.015**	R^2	0.183
		R^2	**0.052 (+)**	0.083 (-)	0.001 (+)	0.045 (+)	0 .004 (-)	**0.096 (+)**		
Fish	Assemblage	P	**0.001**	**<0.001**	0.007	0.006	**0.313**	0.002	R^2	0.147
		R^2	**0.056**	**0.064**	0.043	0.044	**0.022**	0.049		
	Richness	P	0.976	**0.007**	**0.005**	**0.016**	**0.679**	0.035	R^2	0.251
		R^2	<0.001 (+)	**0.121 (+)**	**0.127 (-)**	**0.098 (-)**	**0.003 (+)**	0.077 (-)		
Mollusca	Assemblage	P	**<0.001**	**<0.001**	**<0.001**	0.023	0.068	**<0.001**	R^2	0.197
		R^2	**0.057**	**0.101**	**0.081**	0.033	0.028	**0.051**		
	Richness	P	**0.058**	0.750	0.391	0.730	0.248	0.465	R^2	0.061
		R^2	**0.061 (+)**	0.013 (+)	0.019 (-)	0.002 (+)	0.024 (-)	0.010 (+)		
Universal	Assemblage	P	**0.045**	**0.001**	**0.001**	0.026	0.010	0.014	R^2	0.140
		R^2	**0.034**	**0.061**	**0.059**	0.038	0.043	0.043		
	Richness	P	**0.299**	**0.045**	0.165	0.286	**0.044**	0.578	R^2	0.212
		R^2	**0.019 (+)**	**0.067 (-)**	0.034 (+)	0.020 (+)	**0.068 (+)**	0.006 (+)		

Bolded type indicates abiotic variables that belong to the most parsimonious model as selected using the AIC

+ or – indicate the direction of the relationship

https://doi.org/10.1371/journal.pgen.1007943.t002

Conclusion

A recent editorial on marine monitoring [53] argued for a pressing need to make the shift from site-specific approaches to a functional, whole-sea system of monitoring. Here we show that eDNA metabarcoding is capable of responding to this challenge. Multi-year sample sets appropriate for eDNA analysis have not been previously available. Had this study been limited to a single point in time or even over the course of a year, where the longer-term patterns of change would be missed. Our study included two 'marine heatwave' periods and these data demonstrated that, using an effective eDNA metabarcoding toolkit, ecologically significant trends can be identified in response to a known environmental perturbation.

The biodiversity detected by our multi-assay eDNA metabarcoding 'tool kit' was vast, and while many barcodes could be assigned within the existing taxonomic framework, almost as many could not. While it could be argued that indicator species/OTUs should perhaps be the primary focus for taxonomic scrutiny employing both morphology and genetics, it is clear that as databases and assays improve, so too will the power of eDNA to identify the taxa present in complex ecosystems like this one. The results highlighted both the importance of collecting time-stamped samples (i.e. environmental biobanks [54]) and the significance of multi-gene metabarcoding for the long-term monitoring of marine ecosystems. For example, had only the universal 18S marker been used, much of the genetic depth of information would have been lost. While the 18S markers are typically longer and produce results across a broad range of taxa, it is more conserved than other barcodes and often results must be confined to a family level of identification. The study illustrates the need to balance the cost of the multi-marker approach with the amount of data that can be generated. The future implications of this data are that eDNA will generate much-needed baseline biotic data, and identify disturbance gradients, recovery profiles and potential 'biotic tipping points'.

Materials and methods

Sampling

All sampling took place at the Rottnest Island National Reference Station (NRS), an Integrated Marine Observing System (IMOS [6]) site, Western Australia (Fig 1A). The site is situated at the midpoint of the sub-tropical zone of the Leeuwin current, approximately 20 km off the southwest coast of Western Australia. Abiotic sampling has occurred regularly at this site since 1951 and biological sampling by the IMOS program since 2008 [6]. The plankton sampling regime was instigated at this time and historically three separate monthly samples were taken; one for morphological analysis; one for biomass measurements and a third tow for later DNA analysis. We were provided access to these final samples.

Vertical plankton tows were taken on 55 occasions from October 2009 to January 2015, from the same site, in an almost regular monthly regime (Fig 1B). A 0.6 m wide, 3 m long drop net [55] with a100 μm mesh, which free falls at 1 ms^{-1}, was dropped for 45 s. The seabed depth at the Rottnest Island sampling site is 50 m, so this sampling covered 90% of the water column. Plankton was collected on the downward fall; the motion of retrieval closes the net for the upward haul. The nets are washed in fresh water (with detergent if clogged), hung out to dry and stored dry between monthly sampling.

Samples were washed down and concentrated at the codend of the drop net and transferred into a sample jar using seawater. Samples were packed on ice until placed in long-term storage at -80˚C immediately after return to the laboratory. Samples were later subsampled for this study and the sub-samples preserved at -20˚C prior to DNA extraction.

DNA extraction

Each plankton sample was homogenised, using a hand-held blender (OMNI Tip Homogenizer) and a hard tissue probe. About 20 μL of the resulting slurry was digested and extracted using DNAeasy Blood and Tissue kit (Qiagen) following the tissue protocol and a 2 x 100 μL elution in AE buffer. An extraction control was created during this phase. Extracts were stored at -20˚C.

Metabarcoding assay design

Over 20 group-specific PCR amplicon metabarcode assays were tested for use in this study. Sequences used for *in silico* assay design were downloaded from the National Center for

Biotechnology Information (NCBI) GenBank database [56]. Database coverage was limited across all genes, so in most instances the cytochrome oxidase I (COI) gene provided the best option for metabarcoding.

Sequences were aligned in Geneious Version R8 and consensus sequences were derived from these alignments [57]. Sequences were examined for relatively conserved regions flanking 100-200bp hyper-variable targets (S4 Fig). This examination resulted in the creation of several new metabarcoding assays. These assays, along with some that were previously described, were then tested against 20 pilot plankton samples to determine which assays, when combined, produced the broadest coverage of taxa found within zooplankton (S16 Table). From these, eight assays, including five targeting COI (predominately, three for different copepods and one each for molluscs and cnidarians), one targeting 18S rRNA ("universal") and two targeting16S rRNA (one each for actinopterygii and malacostraca), were selected for use in this study (S1 Table).

The 55 DNA extracts were assessed using qPCR for their response to each of the eight assays, which were applied to each sample's neat extract and two dilutions (1/10 and 1/100). Extraction, non-template and positive controls (where available) were included for each assay. Each reaction comprised: 1 x Taq Gold buffer (Applied Biosystems [ABI], USA), 2 nM MgCl2 (ABI, USA), 0.4 mg/mL BSA (Fisher Biotec, Australia), 0.25 mM dNTPs (Astral Scientific, Australia), 0.4 μM each of forward and reverse primers (Integrated DNA Technologies, Australia), 0.6 μL of 1/10,000 SYBR Green dye (Life Technologies, USA), 1 U of *Taq* polymerase Gold (ABI, USA), 2 μL of DNA, and made up to 25 μL with PCR grade water. PCR conditions for all reactions included 95˚C for 10 min followed by 50 cycles of 95˚C for 30 sec, T_a (S1 Table) for 30 sec and 72˚C for 45 sec, with a final extension of 72˚C for 10 min. All reactions were set up in an ultra-clean laboratory used for trace and environmental DNA.

Library builds & sequencing

Fusion tagged primers incorporating specific unique combinations of six to eight base pair MID (Multiplex IDentifier) tags, assay specific primers and Illumina adaptor sequences were assigned, in duplicate, to each DNA extract (and any negative control that produced a positive result during qPCR) in a single PCR step (giving a total of over 400 unique MID tagged combinations). Many samples are multiplexed within a single library and the MID tags allow for later separation and assignment of the individual sequences to their specific assays and samples. To prevent cross contamination within the NGS workflow, the MID tag primer combinations had not been used previously for marine samples and were not reused. Conditions for the fusion tagged PCR reactions were identical to the qPCR (above) and were carried out in duplicate, using the appropriate dilution determined by the qPCR. Reactions were monitored for efficient amplification by scrutinising qPCR dynamics. Tagged amplicons were combined in roughly equimolar concentrations to produce multiplexed sequencing libraries. On each library the fusion tags were not 'saturated', meaning that, while there are ten reverse tags to every forward tag, each run allowed for several unused forward and reverse combinations. If unused tag combinations are subsequently detected after sequencing, the tagging process is repeated to ensure there is no tag cross over. The libraries were then size-selected using a Pippin Prep (Sage Sciences, USA) instrument and quantified using a Lab Chip (PerkinElmer, USA). All sequencing was performed using Illumina's MiSeq following the manufacturer's protocol with the exception of the use of custom sequencing primers and with 20 pM PhiX, on either a Standard or Nano flow cell and 300–500 cycle kits.

Taxonomic assignment

Sequences were assigned to the appropriate samples by their MID tags using Geneious R8 [57]. Initial filtering steps included ensuring the MID tags, gene specific primers and

sequencing adapters, were all present in each sequence without error. Those sequences not matched were discarded from future analyses. The primers, adaptors and MID tags were removed from each of the sequences that passed these criteria, which were then filtered using a fastq filter (E_max > 0.5—USEARCH v8 [58]).

To increase the robustness of the data set, sequences were then separated into groups of unique sequences using USEARCH v8 [58]. Of these sequences, any group which contained < 1% of the total number of unique sequences was discarded—the filtered data are available for download on Data Dryad: doi:10.5061/dryad.sc673ds. This process, which may eliminate low abundance taxa, is conservative in that it ensures the removal of possible erroneous amplicons. Amplicons that passed the second filtering processes were queried against the National Center for Biotechnology Information (NCBI) GenBank nucleotide database [59] using BLASTn (Basic Local Alignment Search Tool [60]) with the default parameters and a reward of value of 1.

The search output files were imported into MEGAN v5 (METaGenome ANalyzer [61]) and visualised using the LCA (lowest common ancestor) parameters: min bitscore 100.0, and reports restricted to the best 5% of matches. Taxonomic assignment was considered only when the entire length of the query sequence matched the reference database. Taxonomic hierarchy was determined using the World Register of Marine Species [62]. Negative controls were all found to be clear with the exception of the 18S Universal assay, which showed some fungal contamination.

Production of Operational Taxonomic Units (OTUs)

Clustering of similar sequences to produce OTUs was performed with USEARCH v8 [58]. The OTUs were formed from all filtered sequences from each assay using a 97% similarity threshold across all samples. The procedure also removed any potential chimeric sequences and any groups of unique sequences with an abundance of < 0.1% of the total number of unique sequences across all samples. Sequences discarded during this process were then mapped back on to existing OTUs to ensure the inclusion of all relevant data and those amplicons, which could not be mapped, were discarded. The OTUs were then assigned to the samples that they originated from and were converted to a presence/absence matrix. This approach also minimises any data misrepresentations as a result of potential unequal sequence amplification from marker choice or tag bias. The OTUs were statistically analysed in response to both temporal and abiotic factors.

Statistical analysis

Statistical analyses, were performed using PERMANOVA+ [42] add on for Primer 7 [63] and R [64] with labdsv [45], and vegan [65]. The analyses were performed on the presence/absence OTU data matrix for the sequences obtained for each assay, thus allowing for all available genetic information to be taken into consideration. A total of 55 samples were used for analysis. The initial Pearson's correlation test of the number of sequences produced by each assay, at each time point, and the number of OTUs was performed in R [64].

To prevent the inclusion of 'outliers' that might skew the results, the sequences for each assay were filtered to remove any OTUs that occurred only once in the study and also any samples that contained only one OTU. The richness and assemblage (genetic diversity) data for each sample were then examined using multivariate methods (PERMANOVA [66]) to test time-based relationships such as heatwave, seasonality and inter-annual effects). Annual and seasonal effects were tested using a nested design with three factors: Year (fixed, 5 levels), Season (nested in Year, random), and Month (Nested in Season, random). Tests for heatwave

effects were conducted using a single factor (fixed, either 5 month or 17 month heatwave window) with three levels (before, during, after). To illustrate these patterns, two-dimensional nonmetric multidimensional scaling (nMDS) plots were formed in R (package vegan).

The indicator species that were characteristic of years, seasons, and heatwave events were identified using indval analyses in R (package labdsv). The indval indicator value is calculated using a combination of the fidelity of an OTU to a time period and the frequency at which it occurs during that same time period. All pairwise comparisons were performed using PERMANOVA.

The role of abiotic variables in explaining variation in both the multivariate OTU assemblage, and the univariate OTU richness was analysed with linear models for each assay. Multivariate analysis was done using distance based linear models (DistLM) in PERMANOVA+. Bray-Curtis similarity matrices were constructed from the presence/absence OTU data. The abiotic variables sea surface temperature (SST) and concentrations of salinity, silicate, nitrate, phosphate, and ammonium were available for selection by the model. The 'best' selection procedure and the AIC selection criteria were used to select the model that best explained the variation in the OTU assemblage that was recorded for each assay. The best alternative models for each number of variables that were within 2 AIC of the selected model were also reported (S15 Table).

Univariate OTU richness was analysed for each assay with generalised linear models (GLMs) fitted in R using the functions glm [64] and glm.nb [52]. The abiotic explanatory variables available were the same as those above. During analysis the distribution of the residuals of each model were plotted and examined to select the appropriate distribution. In all cases the negative binomial distribution with a log link was used [67]. The model with the lowest AIC was selected using the best of both forward and backward selection procedures. Models within 2AIC of the selected model were also reported. To aid in the interpretation of the relationship between each abiotic variable and the OTU assemblage composition and richness were also calculated and reported for each abiotic variable.

Supporting information

S1 Table. The metabarcoding PCR assays used in this study.
(PDF)

S2 Table. Number of Arthropoda detections in Rottnest Island zooplankton samples by each assay.
(PDF)

S3 Table. Number of Chordata detections in Rottnest Island zooplankton samples by each assay.
(PDF)

S4 Table. Number of Mollusca detections in Rottnest Island zooplankton samples by each assay.
(PDF)

S5 Table. Number of Cnidaria detections in Rottnest Island zooplankton samples by each assay.
(PDF)

S6 Table. Number of Echinodermata detections in Rottnest Island zooplankton samples by each assay.
(PDF)

S7 Table. Number of other Animalia taxa detections in Rottnest Island zooplankton samples by each assay.
(PDF)

S8 Table. Pairwise analysis of seasonal OTU richness & assemblage, t statistics included for significant results (t)—PERMANOVA+[7].
(PDF)

S9 Table. Indicator species analysis for seasonal variation—*Indval* [8].
(PDF)

S10 Table. Pairwise analysis of yearly OTU richness & assemblage, t statistics included for significant results (t)—PERMANOVA+[7].
(PDF)

S11 Table. Indicator species analysis for yearly variation—*Indval* [8].
(PDF)

S12 Table. Pairwise analysis of the OTU richness & assemblage between before, during and after the heatwaves, t statistics included for significant results (t)—PERMANOVA +[7].
(PDF)

S13 Table. Indicator species analysis for five-month heatwave variation—*Indval* [8].
(PDF)

S14 Table. Indicator species analysis for 17-month heatwave variation—*Indval* [8].
(PDF)

S15 Table. Alternative linear models for Assemblage and Richness.
(PDF)

S16 Table. Metabarcoding PCR assays developed and tested for this study.
(PDF)

S1 Fig. Example of typical rarefaction curves. These were produced, using all eight assays, from a single sample taken on May 23 2012
(EPS)

S2 Fig. Number of sequences (beige—right axis) and number of OTUs (red—left axis), per sample, for each assay. The assays showed a range of correlations between sequencing depth and the number of OTUs produced. No correlation was detected in the 18S and Crustacea 16S assays, while the Fish 16S showed a weak but non-significant correlation. The COI assays all produced moderate correlations.
(EPS)

S3 Fig. Annual sea surface temperature anomalies from 1900–2017. The 2011 and 2012 heatwave events produce the two highest peaks—Extracted from the Bureau of Meteorology time series graphs [9].
(PDF)

S4 Fig. Example of a consensus alignment used to create the assays used in this study. This COI alignment resulted in the Copepod 3 assay [10].
(EPS)

S1 Text. References used for supporting information.
(PDF)

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References

1. Beaugrand G, Reid PC, Ibanez F, Lindley JA, Edwards M. Reorganization of North Atlantic marine copepod biodiversity and climate. Science. 2002; 296(5573):1692–4. https://doi.org/10.1126/science.1071329 PMID: 12040196
2. Molinos JG, Halpern BS, Schoeman DS, Brown CJ, Kiessling W, Moore PJ, et al. Climate velocity and the future global redistribution of marine biodiversity. Nature Climate Change. 2016; 6(1):83–8.
3. Palmer CP. Marine biodiversity and ecosystems underpin a healthy planet and social well-being. UN Chronicle. 2017; 54(2):59–61.
4. Edwards M, Beaugrand G, Hays GC, Koslow JA, Richardson AJ. Multi-decadal oceanic ecological datasets and their application in marine policy and management. Trends in ecology & evolution. 2010; 25(10):602–10.
5. Hays GC, Richardson AJ, Robinson C. Climate change and marine plankton. Trends in ecology & evolution. 2005; 20(6):337–44.
6. Lynch TP, Morello EB, Evans K, Richardson AJ, Rochester W, Steinberg CR, et al. IMOS National Reference Stations: a continental-wide physical, chemical and biological coastal observing system. PLOS ONE. 2014; 9(12):e113652. https://doi.org/10.1371/journal.pone.0113652 PMID: 25517905

7. Warner AJ, Hays GC. Sampling by the continuous plankton recorder survey. Progress in Oceanography. 1994; 34(2):237–56.
8. Rice E, Stewart G. Decadal changes in zooplankton abundance and phenology of Long Island Sound reflect interacting changes in temperature and community composition. Mar Environ Res. 2016; 120:154–65. https://doi.org/10.1016/j.marenvres.2016.08.003 PMID: 27552121
9. Richardson A. Plankton and climate. Elements of Physical Oceanography: A derivative of the Encyclopedia of Ocean Sciences. 2009:397.
10. Hirai J, Tsuda A. Metagenetic community analysis of epipelagic planktonic copepods in the tropical and subtropical Pacific. Marine Ecology Progress Series. 2015; 534:65–78.
11. Kelly P, Clementson L, Davies C, Corney S, Swadling K. Zooplankton responses to increasing sea surface temperatures in the southeastern Australia global marine hotspot. Estuarine, Coastal and Shelf Science. 2016; 180:242–57.
12. Edwards M, Richardson AJ. Impact of climate change on marine pelagic phenology and trophic mismatch. Nature. 2004; 430(7002):881–4. https://doi.org/10.1038/nature02808 PMID: 15318219
13. Mackas DL, Greve W, Edwards M, Chiba S, Tadokoro K, Eloire D, et al. Changing zooplankton seasonality in a changing ocean: Comparing time series of zooplankton phenology. Progress in Oceanography. 2012; 97–100:31–62.
14. Chiba S, Tadokoro K, Sugisaki H, Saino T. Effects of decadal climate change on zooplankton over the last 50 years in the western subarctic North Pacific. Global Change Biology. 2006; 12(5):907–20.
15. Johnson CL, Leising AW, Runge JA, Head EJH, Pepin P, Plourde S, et al. Characteristics of *Calanus finmarchicus* dormancy patterns in the Northwest Atlantic. ICES Journal of Marine Science. 2008; 65 (3):339–50.
16. Molinero JC, Ibanez F, Souissi S, Chifflet M, Nival P. Phenological changes in the Northwestern Mediterranean copepods *Centropages typicus* and *Temora stylifera* linked to climate forcing. Oecologia. 2005; 145(4):640–9. https://doi.org/10.1007/s00442-005-0130-4 PMID: 15965753
17. Mackas DL, Batten S, Trudel M. Effects on zooplankton of a warmer ocean: Recent evidence from the Northeast Pacific. Progress in Oceanography. 2007; 75(2):223–52.
18. Williams R. Zooplankton of the Bristol Channel and Severn Estuary. Marine Pollution Bulletin. 1984; 15 (2):66–70.
19. Wiafe G, Dovlo E, Agyekum K. Comparative productivity and biomass yields of the Guinea Current LME. Environmental Development. 2016; 17, Supplement 1:93–104.
20. Rakhesh M, Raman AV, Sudarsan D. Discriminating zooplankton assemblages in neritic and oceanic waters: A case for the northeast coast of India, Bay of Bengal. Marine Environmental Research. 2006; 61(1):93–109. https://doi.org/10.1016/j.marenvres.2005.06.002 PMID: 16125769
21. Lindeque PK, Parry HE, Harmer RA, Somerfield PJ, Atkinson A. Next generation sequencing reveals the hidden diversity of zooplankton assemblages. PLOS ONE. 2013; 8(11):e81327. https://doi.org/10.1371/journal.pone.0081327 PMID: 24244737
22. Purcell JE. Jellyfish and ctenophore blooms coincide with human proliferations and environmental perturbations. Annual Review of Marine Science. 2012; 4:209–35. https://doi.org/10.1146/annurev-marine-120709-142751 PMID: 22457974
23. Bucklin A, Lindeque PK, Rodriguez-Ezpeleta N, Albaina A, Lehtiniemi M. Metabarcoding of marine zooplankton: prospects, progress and pitfalls. Journal of Plankton Research. 2016:fbw023.
24. Markle DF, Frost L-A. Comparative morphology, seasonality, and a key to planktonic fish eggs from the Nova Scotian shelf. Canadian Journal of Zoology. 1985; 63(2):246–57.
25. Harvey JBJ, Johnson SB, Fisher JL, Peterson WT, Vrijenhoek RC. Comparison of morphological and next generation DNA sequencing methods for assessing zooplankton assemblages. Journal of Experimental Marine Biology and Ecology. 2017; 487:113–26.
26. Thomsen PF, Kielgast J, Iversen LL, Møller PR, Rasmussen M, Willerslev E. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. PLOS ONE, 2012; 7(8):e41732 https://doi.org/10.1371/journal.pone.0041732 PMID: 22952584
27. Kelly RP, Closek CJ, O'Donnell JL, Kralj JE, Shelton AO, Samhouri JF. Genetic and manual survey methods yield different and complementary views of an ecosystem. Frontiers in Marine Science. 2017; 3:283
28. Stat M, Huggett MJ, Bernasconi R, DiBattista JD, Berry TE, Newman SJ, et al. Ecosystem biomonitoring with eDNA: metabarcoding across the tree of life in a tropical marine environment. Sci Rep. 2017; 7 (1):12240. https://doi.org/10.1038/s41598-017-12501-5 PMID: 28947818

29. Haouchar D, Haile J, McDowell MC, Murray DC, White NE, Allcock RJN, et al. Thorough assessment of DNA preservation from fossil bone and sediments excavated from a late Pleistocene–Holocene cave deposit on Kangaroo Island, South Australia. Quaternary Science Reviews. 2014; 84:56–64.

30. Peters KJ, Ophelkeller K, Bott NJ, Deagle BE, Jarman SN, Goldsworthy SD. Fine-scale diet of the Australian sea lion (*Neophoca cinerea*) using DNA-based analysis of faeces. Marine Ecology. 2014:1–21.

31. Deagle BE, Kirkwood R, Jarman SN. Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces. Molecular Ecology. 2009; 18(9):2022–38. https://doi.org/10.1111/j.1365-294X.2009.04158.x PMID: 19317847

32. Boyer S, Cruickshank RH, Wratten SD. Faeces of generalist predators as 'biodiversity capsules': A new tool for biodiversity assessment in remote and inaccessible habitats. Food Webs. 2015; 3(0):1–6.

33. Berry TE, Osterrieder SK, Murray DC, Coghlan ML, Richardson AJ, Grealy AK, et al. DNA metabarcoding for diet analysis and biodiversity: A case study using the endangered Australian sea lion (*Neophoca cinerea*). Ecol Evol. 2017; 7(14):5435–53. https://doi.org/10.1002/ece3.3123 PMID: 28770080

34. Deagle BE, Clarke LJ, Kitchener JA, Polanowski AM, Davidson AT. Genetic monitoring of open ocean biodiversity: An evaluation of DNA metabarcoding for processing continuous plankton recorder samples. Mol Ecol Resour. 2017.

35. Taberlet P, Brown A., Zinger L., and Coissac E. Environmental DNA for Biodiversity Research and Monitoring Oxford: Oxford University Press; 2018.

36. Roberts CM, McClean CJ, Veron JE, Hawkins JP, Allen GR, McAllister DE, et al. Marine biodiversity hotspots and conservation priorities for tropical reefs. Science. 2002; 295(5558):1280–4. https://doi.org/10.1126/science.1067728 PMID: 11847338

37. Caputi N, Kangas M, Denham A, Feng M, Pearce A, Hetzel Y, et al. Management adaptation of invertebrate fisheries to an extreme marine heat wave event at a global warming hot spot. Ecol Evol. 2016; 6 (11):3583–93. https://doi.org/10.1002/ece3.2137 PMID: 28725352

38. Pearce AF, Feng M. The rise and fall of the "marine heat wave" off Western Australia during the summer of 2010/2011. Journal of Marine Systems. 2013; 111–112:139–56.

39. Wernberg T, Bennett S, Babcock RC, de Bettignies T, Cure K, Depczynski M, et al. Climate-driven regime shift of a temperate marine ecosystem. Science. 2016; 353(6295):169. https://doi.org/10.1126/science.aad8745 PMID: 27387951

40. Alberti A, Poulain J, Engelen S, Labadie K, Romac S, Ferrera I, et al. Viral to metazoan marine plankton nucleotide sequences from the Tara Oceans expedition. Scientific data. 2017; 4:sdata201793.

41. Richardson AJ, Uribe-Palomino J, Slotwinski A, Coman F, Miskiewicz AG, Rothlisberg PC, et al. Coastal and marine zooplankton: identification, biology and ecology. Plankton: A guide to their ecology and monitoring for water quality: CSIRO publishing; 2019 (in press).

42. Anderson M, Gorley RN, Clarke RK. Permanova+ for Primer: Guide to Software and Statistical Methods: Primer-E Limited; 2008.

43. Stoeckle MY, Soboleva L, Charlop-Powers Z. Aquatic environmental DNA detects seasonal fish abundance and habitat preference in an urban estuary. PLOS ONE. 2017; 12(4):e0175186. https://doi.org/10.1371/journal.pone.0175186 PMID: 28403183

44. Sigsgaard EE, Nielsen IB, Carl H, Krag MA, Knudsen SW, Xing Y, et al. Seawater environmental DNA reflects seasonality of a coastal fish community. Marine Biology. 2017; 164(6).

45. Roberts DW. labdsv: Ordination and Multivariate Analysis for Ecology. 2016;R package version 1.8–0.

46. ALA. Atlas of Living Australia website http://www.ala.org.au 2016

47. Caputi N, Fletcher WJ, Pearce A, Chubb CF. Effect of the Leeuwin Current on the Recruitment of Fish and Invertebrates along the Western Australian Coast. Marine and Freshwater Research. 1996; 47 (2):147–55.

48. Pearce A, Hutchins B, Hoschke A, Fearns P. Record high damselfish recruitment at Rottnest Island, Western Australia, and the potential for climate-induced range extension. Regional Studies in Marine Science. 2016; 8:77–88.

49. Lenanton RCJ, Dowling CE, Smith KA, Fairclough DV, Jackson G. Potential influence of a marine heatwave on range extensions of tropical fishes in the eastern Indian Ocean—Invaluable contributions from amateur observers. Regional Studies in Marine Science. 2017; 13:19–31.

50. Caputi N, Jackson, G. and Pearce, A. The marine heat wave off Western Australia during the summer of 2010/11–2 years on. Fisheries Research Report No 250; 2014.

51. George Jennifer A, Lonsdale Darcy J, Merlo Lucas R, Gobler Christopher J. The interactive roles of temperature, nutrients, and zooplankton grazing in controlling the winter–spring phytoplankton bloom in a temperate, coastal ecosystem, Long Island Sound. Limnology and Oceanography. 2015; 60(1):110–26.

52. Venables W, Ripley B. Random and mixed effects. Modern applied statistics with S: Springer; 2002. p. 271–300.

53. Borja A, Elliott M, Andersen JH, Cardoso AC, Carstensen J, Ferreira JG, et al. Good Environmental Status of marine ecosystems: what is it and how do we know when we have attained it? Mar Pollut Bull. 2013; 76(1–2):16–27. https://doi.org/10.1016/j.marpolbul.2013.08.042 PMID: 24054784

54. Jarman SN, Berry O, Bunce M. The value of environmental DNA biobanking for long-term biomonitoring. Nature Ecology & Evolution. 2018; 2(8):1192–3.

55. Heron AC. A vertical free fall plankton net with no mouth obstructions. Limnology and Oceanography. 1982; 27(2):380–3.

56. Benson DA, Karsch-Mizrachi I, Clark K, Lipman DJ, Ostell J, Sayers EW. GenBank. Nucleic Acids Res. 2012; 40(Database issue):D48–53. https://doi.org/10.1093/nar/gkr1202 PMID: 22144687

57. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012; 28(12):1647–9. https://doi.org/10.1093/bioinformatics/bts199 PMID: 22543367

58. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010; 26 (19):2460–1%@ 1367–4803. https://doi.org/10.1093/bioinformatics/btq461 PMID: 20709691

59. Benson DA, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW. GenBank. Nucleic Acids Research. 2014; 42 (Database issue):D32–D7. https://doi.org/10.1093/nar/gkt1030 PMID: 24217914

60. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. Journal of Molecular Biology. 1990; 215(3):403–10. https://doi.org/10.1016/S0022-2836(05)80360-2 PMID: 2231712

61. Huson DH, Mitra S, Ruscheweyh HJ, Weber N, Schuster SC. Integrative analysis of environmental sequences using MEGAN 4. Genome Res. 2011; 21:1552–60. https://doi.org/10.1101/gr.120618.111 PMID: 21690186

62. World Register of Marine Species (WoRMS) [Internet]. WoRMS Editorial Board. 2018 [cited 2018-04-11]. Available from: http://www.marinespecies.org.

63. Clarke K, Gorley R. Getting started with PRIMER v7. PRIMER-E: Plymouth, Plymouth Marine Laboratory. 2015.

64. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria Vienna, Austria2015 [Available from: http://www.R-project.org/.]

65. Oksanen J, Guillaume Blanchet F, Friendly M, Kindt R, Legendre P, McGlinn D, et al. vegan: Community Ecology Package. R package version 2.3–0. http://CRAN.R-project.org/package=vegan 2016.

66. Anderson MJ. A new method for non-parametric multivariate analysis of variance. Austral ecology. 2001; 26(1):32–46.

67. Zuur A, Ieno E, Walker N, Saveliev A, Smith G. Mixed effects models and extensions in ecology with R. New York: Springer. 574 p. 2009.

Assessing the trophic ecology of top predators across a recolonisation frontier using DNA metabarcoding of diets

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ABSTRACT: Top predator populations, once intensively hunted, are rebounding in size and geographic distribution. The cessation of sealing along coastal Australia and subsequent recovery of Australian *Arctocephalus pusillus doriferus* and long-nosed *A. forsteri* fur seals represents a unique opportunity to investigate trophic linkages at a frontier of predator recolonisation. We characterised the diets of both species across 2 locations of recolonisation, one site an established breeding colony, and the other, a new but permanent haul-out site. Using DNA metabarcoding, high taxonomic resolution data on diets was used to inform ecological trait-based analyses across time and location. Australian and long-nosed fur seals consumed 76 and 73 prey taxa, respectively, a prey diversity greater than previously reported. We found unexpected overlap of prey functional traits in the diets of both seal species at the haul-out site, where we observed strong trophic linkages with coastal ecosystems due to the prevalence of benthic, demersal and reef-associated prey. The diets of both seal species at the breeding colony

were consistent with foraging patterns observed in the centre of their geographic range regarding diet partitioning between predator species and seasonal trends typically observed. The unexpected differences between sites in this region and the convergence of both predators' effective ecological roles at the range-edge haul-out site correlate with known differences in seal population densities and demographics at these and other newly recolonised locations. This study provides a baseline for the diets and trophic interactions for recovering fur seal populations and from which to understand the evolving ecology of predator recolonisation.

KEY WORDS: DNA metabarcoding · Trophic ecology · Predator–prey interactions · Recolonisation · Fur seals · *Arctocephalus forsteri* · *Arctocephalus pusillus doriferus* · Otariid

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Real-time PCR detection of *Didemnum perlucidum* (Monniot, 1983) and *Didemnum vexillum* (Kott, 2002) in an applied routine marine biosecurity context

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Abstract

Prevention and early detection are well recognized as the best strategies for minimizing the risks posed by non-indigenous species (NIS) that have the potential to become marine pests. Central to this is the ability to rapidly and accurately identify the presence of NIS, often from complex environmental samples like biofouling and ballast water. Molecular tools have been increasingly applied to assist with the identification of NIS and can prove particularly useful for taxonomically difficult groups like ascidians. In this study, we have developed real-time PCR assays suited to the specific identification of the ascidians *Didemnum perlucidum* and *Didemnum vexillum*. Despite being recognized as important global pests, this is the first time specific molecular detection methods have been developed that can support the early identification and detection of these species from a broad range of environmental sample types. These fast, robust and high-throughput assays represent powerful tools for routine marine biosecurity surveillance, as detection and confirmation of the early presence of species could assist in the timely establishment of emergency responses and control strategies. This study applied the developed assays to confirm the ability to detect Didemnid eDNA in water samples. While previous work has focused on detection of marine larvae from water samples, the development of real-time PCR assays specifically aimed at detecting eDNA of sessile invertebrate species in the marine environment represents a world first and a significant step forwards in applied marine biosecurity surveillance. Demonstrated success in the detection of *D. perlucidum* eDNA from water samples at sites where it could not be visually identified suggests value in incorporating such assays into biosecurity survey designs targeting Didemnid species.

Keywords: *Didemnum*, environmental DNA, introduced species, invasive, marine biosecurity monitoring, pests, real-time PCR

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Introduction

Marine nonindigenous species (NIS) can pose a significant threat to coastal ecosystems through their potential to decrease endemic biodiversity and modify habitats as well as threaten marine industries and infrastructure (Rilov & Crooks 2009). NIS are commonly transported and introduced through hull biofouling and ballast water, and represent an increasing threat due to globalization and increases in worldwide shipping activities.

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The total economic cost of an NIS incursion is very difficult to determine because it can include not only direct costs related to reductions in economic output but also indirect costs via damage to coastal infrastructure, social implications, risks to human health and environmental impacts, which can be more difficult to quantify. What is clear, however, is that once an incursion has occurred, eradication of a NIS is very costly and often ineffective (Pimentel *et al.* 2000; Bax *et al.* 2003; Pochon *et al.* 2013). Prevention and early detection are well recognized as the best strategies for minimizing the risks posed by NIS that have the potential to become marine pests (Bax *et al.* 2001; Simberloff 2001; Hulme 2006). Central to this is the

ability to rapidly and accurately identify the presence of NIS, often from complex environmental samples like biofouling and ballast water (Bott *et al.* 2010). In general, early detection of NIS can be problematic, as some species require highly specialized taxonomic expertise and morphological identification at early life stages is often challenging, particularly if samples are poorly preserved (Darling & Blum 2007; Darling & Mahon 2011).

In an attempt to address some of these challenges, molecular tools have been increasingly applied to assist with the identification of NIS. These include DNA barcoding, real-time PCR and, more recently, metabarcoding (Darling & Blum 2007; Bott *et al.* 2010; Comtet *et al.* 2015; Zaiko *et al.* 2015). DNA barcoding involves the PCR amplification and sequencing of a diagnostic DNA region or gene (e.g. COI) and analysing that sequence against a reference database such as GenBank (Benson *et al.* 2013) or BoLD (Barcode of Life Data System, Ratnasingham & Hebert 2007). Such methodologies require only small amounts of tissue, allow for the identification of species at all development stages and are becoming standardized across a wide range of taxa (Hebert *et al.* 2003). DNA barcoding has developed, during the last decade, into an efficient and affordable methodology routinely applied to complement taxonomic identification of individual specimens of suspected NIS (Armstrong & Ball 2005; Bott *et al.* 2010; Comtet *et al.* 2015).

Real-time PCR is a method that, in addition to primers, includes fluorescent probes in the reaction which bind to the target species DNA. The species-specific detection occurs in 'real-time' during the reaction, reducing the time arising from the postprocessing of samples and increasing sensitivity as compared to the end-point detection in agarose gels following conventional PCR. The probe fluorescence intensity is measured during the exponential amplification phase when it rises above the background level or critical threshold (Ct), and is directly correlated with initial template quantity. For this reason, real-time PCR can also be used quantitatively, with lower Ct values corresponding to an amplifiable higher DNA amount (Valasek & Repa 2005). The decreased processing time combined with the increased specificity and sensitivity of real-time PCR has motivated development of this technology for the detection of target NIS from complex environmental samples (Bott *et al.* 2010; Smith *et al.* 2012a; Loh *et al.* 2014).

The use of molecular methods for biosecurity applications has been gaining momentum worldwide. In Australia and New Zealand, PCR and real-time PCR assays developed and adopted by biosecurity agencies have been aimed at detecting larval stages of NIS in the plankton communities of ballast water and commercial harbours (Deagle *et al.* 2003; Gunasekera *et al.* 2005; Smith *et al.* 2012a; Wood *et al.* 2013). In Canada, specific PCR assays have also been developed for the confirmation and detection of invasive tunicates species at multiple life stages (Stewart-Clark *et al.* 2009, 2013). In addition to the targeted larval or gamete life stages of planktonic organisms, sources of NIS DNA present in the environment also include mucus, tissue, waste and free DNA. In the marine environment, environmental DNA (eDNA) has not been historically targeted by NIS detection methodologies until the recent advent of sensitive metabarcoding assays based on emerging high-throughput sequencing (HTS) technology (Thomsen & Willerslev 2015). The ability to amplify thousands of DNA sequences (including a significant amount of trace DNA) from water samples has allowed researchers to detect eDNA from a variety of marine species including dolphins and fish (Foote *et al.* 2012; Thomsen *et al.* 2012). The background biodiversity information and sensitivity associated with the 'deep-sequencing' capacity of metabarcoding technologies have long looked promising for the early detection of marine NIS (Darling & Blum 2007; Bott *et al.* 2010; Comtet *et al.* 2015; Zaiko *et al.* 2015). Despite reduction in costs and increased ease of access to HTS platforms, the successful application of metabarcoding to marine biosecurity is still greatly hindered by the lack of primers able to amplify a suitable shorter size read, diagnostic for the highly diverse taxa of largely invertebrate NIS (Pochon *et al.* 2013).

Ascidians are common among biofouling communities and are among the taxa with the highest reported record of introduced species worldwide (Lambert 2002; López-Legentil *et al.* 2015; Pagad *et al.* 2015). Non-native Didemnid ascidians, including the recognized marine pests *Didemnum perlucidum* and *Didemnum vexillum*, are considered a threat to Western Australia (WA) and are thus included on the WA Prevention List for Introduced Marine Pests (2014). *D. perlucidum* is an invasive colonial ascidian that has been introduced to many locations worldwide (Lambert 2002; Dias *et al.* 2016). In WA, it has become well established on the infrastructure of ports, marinas and aquaculture facilities and on seagrass meadows of the Swan River (Bridgwood *et al.* 2014; Muñoz & McDonald 2014; Simpson *et al.* 2016). Although *D. perlucidum* eradication is deemed unfeasible from many areas where it has become established, all suspected new detections of this species must be reported to the WA Government Department of Fisheries (DoF) so that its distribution can be tracked and its further spread potentially limited. *D. vexillum* has also been described as a marine pest around the world, aggressively overgrowing native species and causing environmental and economic damage (Stefaniak *et al.* 2009), but to date has not been recorded in Australia. Routine surveillance is required to prevent future incursions of this species. Effectively managing the risk of introduction and spread of

Didemnid species requires development of a fast and accurate method of confirming specimen identity given that these species are often difficult to identify visually. Colonies are variable in colour and morphology, as has been shown for both species (Stefaniak *et al.* 2009; Bridgwood *et al.* 2014), and taxonomic identification is challenging, provided by only a few experts outside Australia. The development of methods suited to the rapid detection of Didemnid species, and particularly *D. perlucidum* and *D. vexillum*, from surveillance programs remains a priority for the effective management of the risk posed by these species to WA.

To address this priority, the aims of this study were to (i) develop real-time PCR assays suited to the rapid identification of *D. perlucidum* and *D. vexillum*; (ii) test the suitability of real-time PCR for detecting *Didemnum perlucidum* eDNA from water samples; and (iii) evaluate the potential of integrating these methods into current routine marine biosecurity monitoring regimes.

Materials and methods

Vouchered specimen and larvae collection for primer testing

Vouchered specimens of many Didemnid species and larvae from D. *perlucidum* were obtained to confirm the specificity and sensitivity of the primer sets prior to applying them to water samples. The DoF molecular laboratory was given access to taxonomically confirmed samples of DNA extracted from whole colonial tissue of *D. perlucidum* and lookalike Didemnid species collected as part of introduced marine pest (IMP) monitoring along the WA coast during 2011–2012 (Bridgwood *et al.* 2014; Table 1). Didemnid DNA barcoding was performed as described in Bridgwood *et al.* (2014), and all WA *D. perlucidum* sequences were a 100% haplotype match to JQ731735, obtained from the Swan River from this species first detection in WA in 2010 (Smale & Childs 2012). A DNA sample of a second distinct haplotype of *D. perlucidum* (GenBank JQ731740) was provided by the New South Wales (NSW) Department of Primary Industries. Further, a taxonomically vouchered *D. vexillum* DNA sample was made available from the DoF Taxonomic and Molecular Reference Collection (BoLD OZIMP002-15).

Larvae were collected from colonies of confirmed *D. perlucidum* collected at Hillarys Boat Harbour (HBH) and stored in ethanol during the reproductive peak in summer (Muñoz *et al.* 2015). Samples consisting of one larva and pools of two and five larvae were sorted using a dissection microscope and transferred to Eppendorf tubes with 70% ethanol, in triplicate. Ethanol was removed from the larvae using a pipette and DNA was extracted from all larval samples using a Favorgen FavorPrep Tissue Genomic DNA Extraction Mini Kit, following the manufacturer's instructions (Fisher Biotec). A blank filter control and a template-free extraction control were included, and all extracts were resuspended in 50 μL elution buffer. All extracts were stored at −20 °C until further use.

Table 1 List of species from which samples were obtained to test assay specificity and collection location; haplotype (Hap) 1 is represented by GenBank JQ731735 and Hap 2 by GenBank JQ731740

Species	Location	Hap	Dper	Dvex
Didemnum perlucidum	Cygnet Bay, Western Australia	1	✓	×
	Dampier, Western Australia	1	✓	×
	Barrow Island, Western Australia	1	✓	×
	Geraldton, Western Australia	1	✓	×
	Hillarys, Western Australia	1	✓	×
	Fremantle, Western Australia	1	✓	×
	Henderson, Western Australia	1	✓	×
	Busselton, Western Australia	1	✓	×
	Swan River, Western Australia	1	✓	×
	Twofold Bay, New South Wales	2	✓	×
Didemnum vexillum	Port Nelson, New Zealand		×	✓
Didemnum patulum	Cygnet Bay, Western Australia		×	×
Didemnum incanum	Albany, Western Australia		×	×
Lissoclinum fragile	Port Hedland, Western Australia		×	×
Didemnum sp.	Queenscliff Marina, Victoria, Australia		×	×
Didemnum sp.	Broome, Western Australia		×	×

A check mark indicates a positive Ct value and '×' a negative result for *D. perlucidum* assay (Dper) and *D. vexillum* assay (Dvex)

Real-time PCR assay development, optimization and testing

The COI gene was targeted for the development of *D. perlucidum*- and *D. vexillum*-specific real-time PCR assays due to an appropriate level of variation within this gene

region and availability of COI sequences for *Didemnum* species in public databases. At the time this work was conducted in 2014, sequences JQ731735 and JQ731740 represented the only two known COI haplotypes for *D. perlucidum*. These sequences were aligned with all known 16 haplotype sequences of *D. vexillum* (Stefaniak *et al.* 2009; Smith *et al.* 2012b) and 17 sequences of another six *Didemnum* species including *D. patulum* (JQ731736-9), *D. fulgens* (JX846617, KF309576), *D. incanum* (JQ692626-8), *D. psammatode* (EU742661), *D. albidum* (EU419432, EU419456) and *D. granulatum* (JQ780669, JQ780673, JQ780675, JQ780687, JQ780688). One primer pair per species was developed that flanked highly variable regions suitable for the design of species-specific TaqMan-MGB probes (Table 2). The primers and probes were designed using the Primer Express version 3.0 software (Applied Biosystems), based on the alignment of all sequences detailed above. To help guarantee the specificity of the method, all designed primer and probe sequences were tested in silico using the similarity-based Basic Local Alignment Search Tool (BLAST) [National Center for Biotechnology Information (NCBI)], to check for potential cross-reaction.

Real-time PCR assay testing and optimization was conducted using both multiplex and single-probe reactions conducted in a final volume of 10 μL containing 1 μL of DNA template, 1× TaqMan® Fast Advanced master mix (Applied Biosystems), and combinations of concentrations of primers and probes (450, 900 and 1350 nM of each primer and 100, 200 and 300 nM of each TaqMan® probe) (Applied Biosystems). Assays were performed on an ABI Step One Plus™ real-time PCR system using a cycling profile of 50 °C for 2 min (UNG incubation) and 95 °C for 20 s (DNA polymerase activation) followed by 45 cycles of 95 °C for 1 s (denaturation) and 60, 58, 56 and 54 °C for 20 s (annealing/extension). Assay specificity was tested across DNA extracts of Didemnid species (Table 1). All experiments included a negative control (no template DNA added). The efficiency of primers and probes, *that is* Efficiency (%) = $[10^{(-1/\text{slope})}] - 1 \times 100$, was assessed using standard curves based on triplicate singleplex reactions conducted on 10-fold dilutions of DNA extracted from *D. perlucidum* and *D. vexillum* colonies. Starting concentrations for the serial dilutions were 88.3 μg/mL *D. perlucidum* and 48.6 μg/mL *D. vexillum*. DNA extracts from confirmed *D. perlucidum* and *D. vexillum* colonies were used as positive controls in all runs. A standard curve based on DNA extracted from the samples of *D. perlucidum* larvae was established to investigate the minimum number of larvae able to be detected by each assay.

Water sampling and DNA extraction

Water sampling was conducted at two locations, Hillarys Boat Harbour (HBH) (31°49′30.70″S, 115°44′07.71″E) and the lower Swan River Estuary (32°0′ 20.43″S, 115°46′ 20.00″E), in the WA Perth metropolitan area (Fig. 1) where *D. perlucidum* is known to be well established (Smale & Childs 2012; Muñoz *et al.* 2015). Water sampling and visual surveys were conducted simultaneously in May, August and December 2014 at eight HBH sites. Five sites were located within the HBH sea walls, and three sites outside the HBH sea walls, namely at the HBH entrance, at the Boy in a Boat Reef sanctuary (no take) zone and at a further-away control site. Water samples were collected by hand from a boat using sterile 100-mL plastic jars (five replicates) and latex gloves, just below the water surface. The presence or absence of *D. perlucidum* was noted during snorkel surveys of the artificial structures neighbouring the five water sampling sites within HBH. Within the Swan River, water samples were collected opportunistically at a single occasion and site in Mosman Bay during January 2015. Visual surveys verified the presence of *D. perlucidum* colonies growing on seagrass on the river bottom at approximately 2.5 m depth. Five replicate water samples were collected from the surface above the colonies, mid-water column (1 m) and at the bottom near *D. perlucidum* colonies (2.5 m deep).

In the laboratory, all the replicate water samples collected from the same site and depth (five samples,

Table 2 List of real-time PCR primers and TaqMan®-MGB probes developed in this study with information on species assay, oligo name, sequence, melting temperature (Tm), GC content (%), length (bp = base pairs) and attributed dye (probes)

Assay	Oligo	Sequence 5′–3′	Tm (°C)	GC (%)	Length (bp)	Dye
Didemnum perlucidum	Dper new F	AGCTCCTGATATAGCATTTCCTCGTTTAAA	63.3	37	30	—
	Dper new R	AGATATTCCTGCTAAATGTAATGAAAAAATAGCTA	61.2	26	35	—
	Dp probe	TAGCTCATTCAAATAGGGCAGTA	69	39	23	FAM
Didemnum vexillum	Dvex new F	TGATTATTACCTTTAATAATCAGAGCTCCAGATA	61	29	34	—
	Dvex new R	AGATATTCTAGCTAAATGTAGAGAAAAAATAACTA	56.2	23	35	—
	Dv probe2*	ACTGTTCATCTAGTTCTAGCTC	69	41	22	VIC

*The Dv probe2 has been designed on the lagging strand.

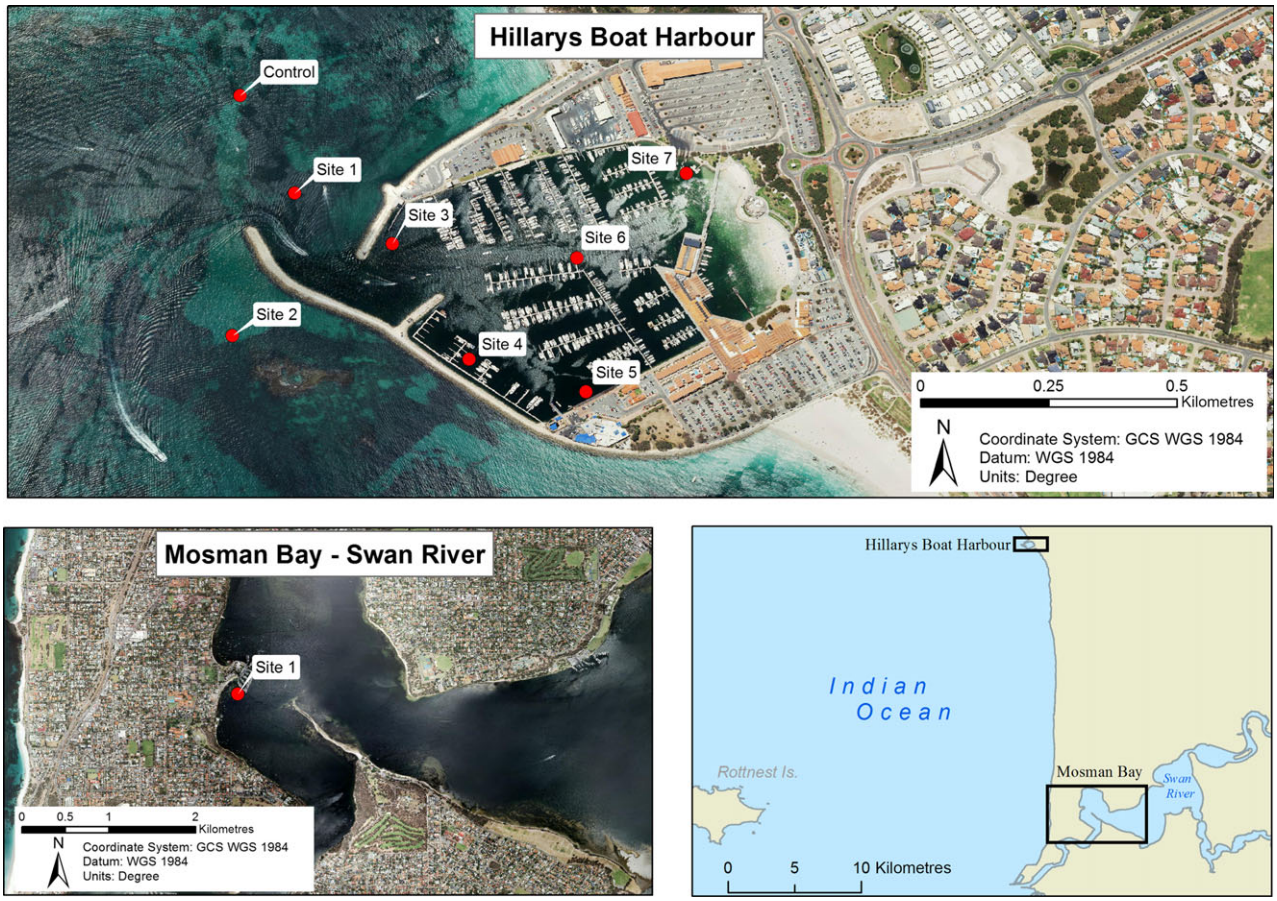

Fig. 1 Map showing eight sampling sites (1–7 and control) at Hillarys Boat Harbour (HBH) and the single site at Mosman Bay in the Swan River, Perth, Western Australia.

100 mL each) were pooled and spiked with *Artemia franciscana*. This is often used as an extraction process control (Giblot-Ducray & Bott 2013). One set of parallel samples was processed without the addition of *Artemia* to ensure that it did not limit the efficacy of the method. The pooled water samples were then filtered using an in-house assembled filtration unit (Fig. 2). The unit was soaked in 10% bleach, rinsed, sprayed with absolute ethanol and dried prior to filtration. A nitrocellulose filter (0.45 μm HA; Merck Millipore®) was placed on each unit using sterilized forceps. A volume of 500 mL of distilled water was passed through the filter prior to each sample as a blank negative control. The entire seawater sample (500 mL) was then filtered through a new filter which was transferred to a 7-mL prefilled Precellys® bead tube and stored at −20 °C until DNA extraction. A Favorgen FavorPrep Tissue Genomic DNA Extraction Mini Kit was used to extract DNA from the filtered samples, following the manufacturer's instructions (Fisher Biotec). The volumes of FATG1, FATG2 buffer, proteinase K and ethanol were increased from the manufacturer's instructions to provide enough reagent volume to fully lyse the whole filter. The filter was homogenized with a Precellys homogenizer (4024 ***g***, 3 × 30 s, 15 s hold). The homogenate was centrifuged, and the supernatant was transferred to a fresh Eppendorf tube. From that point, the protocol followed the manufacturer's instructions. An extraction control (blank filter) was included, and all samples were eluted in 100 μL of elution buffer. All DNA extracts were stored at −20 °C until further use.

Real-time PCR screening of water samples

DNA extracts obtained from all water samples were screened using the developed *D. perlucidum* and *D. vexillum* singleplex real-time PCR assays (Table 2). All reactions were conducted in a final volume of 10 μL containing 1 μL of DNA template, 1× TaqMan® Fast Advanced master mix (Applied Biosystems), 450 or 1350 nM of each primer for *D. perlucidum* and *D. vexillum* assays respectively, and 200 nM of TaqMan® probe (Applied Biosystems). All assays were performed on an ABI Step One Plus™ real-time PCR system using a

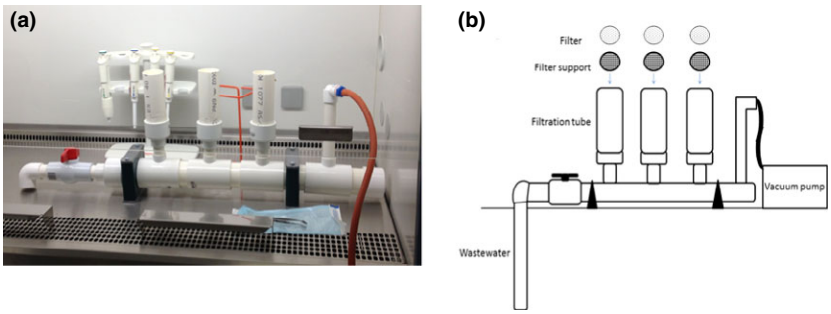

Fig. 2 (a) Photograph of the water multi-sample filtration unit, assembled in-house from cut and adjusted plastic parts, and metal mesh for filter support. Sized for use within laminar flow cabinet (MSC-Advantage; Thermo Fisher) and using a vacuum pump (SparMax); (b) schematic diagram of the filtration unit.

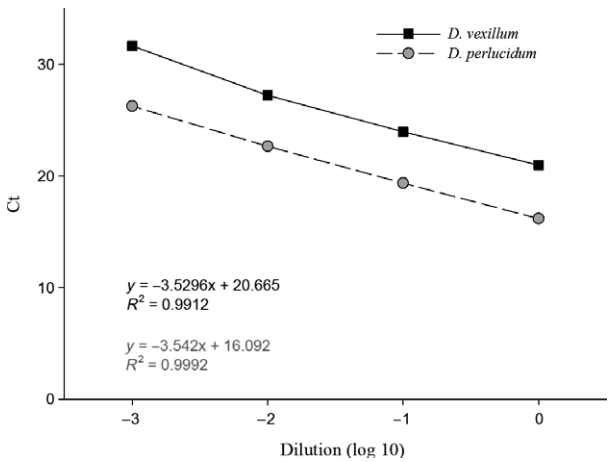

Fig. 3 Efficiency of *D. perlucidum* (Dper) and *D. vexillum* (Dvex) real-time PCR singleplex assays. Average cycle threshold values (Cts) obtained from 10-fold dilutions of total DNA extracted from *D. perlucidum* (neat DNA concentration 88.3 μg/mL) and *D. vexillum* (neat concentration 48.6 μg/mL) colonial tissue. Slope values giving reaction efficiency of TaqMan®-MGB probes for each species are shown on the graphic. Standard deviations (SD) are ±0.04 and ±0.42 for Dper and Dvex, respectively, and are too low to be visualized in the figure.

cycling profile of 50 °C for 2 min (UNG incubation) and 95 °C for 20 s (DNA polymerase activation) followed by 45 cycles of 95 °C for 1 s (denaturation) and 60 or 58 °C for 20 s (annealing/extension) for *D. perlucidum* and *D. vexillum*, respectively. Reactions were conducted in triplicate, and all experiments included an equipment control, extraction control and negative real-time PCR control (no template DNA added).

Results

Real-time PCR assay development, optimization and testing

When *D. perlucidum* and *D. vexillum* assays were multiplexed, the *D. vexillum* assay was strongly inhibited. To obtain accurate, reproducible and comparable results, real-time PCR assay efficiency should be as close to 100% (slope of −3.33) as possible (Pfaffl 2004; Valasek & Repa 2005). When used as singleplex assays, standard curves based on triplicate reactions of 10-fold dilutions of DNA extracted from *D. perlucidum* and *D. vexillum* colonies revealed slopes of −3.35 (efficiency = 98.84%) and −3.52 (efficiency = 92.35%), respectively. There was high correlation between Ct values and dilution factor, $R^2 = 0.991$ and $R^2 = 0.999$ for *D. perlucidum* and *D. vexillum*, respectively (Fig. 3). Triplicate single-probe reactions yielded similar Ct values, with SD ±0.003–0.085 for *D. perlucidum* and SD ±0.099–0.688 for *D. vexillum* dilution series.

Results from in silico evaluation of primer and probe specificity indicated that the designed real-time PCR assays were specific for *D. perlucidum* and *D. vexillum*. This was supported by screening several Didemnid species. All *D. perlucidum* DNA samples generated positive Ct values (Ct range 21.5–35) when screened with the *D. perlucidum* assay. There was no cross-amplification with all other Didemnid species screened (Table 1). Similarly, the *D. vexillum* DNA sample tested positive when screened with the *D. vexillum* assay (Ct range 24.6–31.2), while all other screened Didemnid species were negative (Table 1). The *D. perlucidum* assay was also able to detect all triplicate DNA samples extracted from pools of five larvae (average Ct 25.1 ± 0.05, DNA concentration 0.525 μg/mL), two larvae (average Ct 27.65 ± 0.04, DNA concentration 0.188 μg/mL) and from one larva (average Ct 28.56 ± 0.06, concentration too low to be determined). No detections were obtained from extraction controls or real-time PCR negative controls.

Real-time PCR screening of water samples

Water samples collected in May from HBH and screened using the real-time PCR assay for *D. perlucidum* were all positive with the exception of the control site. Values ranged from Ct 33.3 to Ct 40.2 (Table 3). Visual surveys within the harbour confirmed the presence of *D. perlucidum* at all sites (Fig. 1; Table 3). Sites 1 and 2 outside the HBH sea walls were not visually assessed but were expected to have little to no presence of *D. perlucidum* due to sandy bottoms and the absence of *D. perlucidum*

Table 3 Comparison of *D. perlucidum* detections through visual surveys and average Ct values (±SD) of triplicate reactions of DNA extracted from water samples and screened using the *D. perlucidum* real-time PCR (ND indicates no detection)

		May-14		Aug-14		Dec-14		Jan-15	
Sampling site	Location	Visual	qPCR	Visual	qPCR	Visual	qPCR	Visual	qPCR
Marina 1	Marina entrance		39.8		ND		ND		
Marina 2	Reef		40.2		40.2 ± 1.1		ND		
Marina 3	Refueling Jetty	✓	35.4 ± 0.3	✓	38.7	X	ND		
Marina 4	Jetty Z	✓	34.8 ± 0.2	✓	39.7 ± 1.3	✓	ND		
Marina 5	Wooden Jetty	✓	35.1 ± 0.2	X	38.7	X	ND		
Marina 6	Jetty D	✓	34.9 ± 0.4	X	38.9 ± 1.8	✓	ND		
Marina 7	Boat ramp	✓	33.5 ± 0.01	✓	38.2 ± 0.8	✓	38.1 ± 0.1		
Marina 8	Control Site		ND		ND		ND		
Mosman Bay 1	Surface								ND
Mosman Bay 2	Mid-column 1 m depth								39.1 ± 1.4
Mosman Bay 3	Bottom 2.5 m depth							✓	38.1 ± 0.9

Higher Ct values imply lower concentrations of target DNA

from natural reefs in WA. In August, *D. perlucidum* was visually detected at only three sites, but it was detected by the real-time PCR assay at all sites, excluding the control site and Site 1. Values ranged from Ct 38.2 to Ct 40.2 (Table 3). In December, visual surveys indicated *D. perlucidum* was present at three of five sites, but real-time PCR detections were positive only at Site 7 with a value of Ct 38.1 (Table 3). From the water samples collected at Mosman Bay in January, the real-time PCR assay detected *D. perlucidum* eDNA at the river bottom near the observed colonies (~2.5 m depth) and in the water column (~1 m depth) but did not detect it from the surface (Table 3). All samples spiked with *A. franciscana* were positive for this species without hindering the ability to detect *D. perlucidum*. As expected, there were no *D. vexillum* detections in any of the water samples from the HBH or Mosman Bay. No detections were obtained from equipment, extraction or real-time PCR negative controls.

Discussion

In this study, we have developed rapid, sensitive and species-specific real-time PCR assays suited to the specific identification of *D. perlucidum* and *D. vexillum*. Despite being recognized as important global pests, this is the first time an assay has been developed which is suitable for the fast and high-throughput routine identification and detection of *D. perlucidum* and *D. vexillum* from environmental samples. The ability to detect DNA extracted from as little as one larva and the efficiency values obtained for each assay indicate detections should be robust and reproducible, providing the necessary confidence for the confirmation of these species from tissue samples. The fact that the *D. perlucidum* assay was able to detect not only the commonly found haplotype in WA but also the haplotype reported from the only incursion event to date to the eastern states (Twofold Bay, New South Wales NSW) indicates the assay is suitable to monitor both potential spread of and potential future incursions of *D. perlucidum.* While not tested in the field, the in silico results provide confidence that the *D. vexillum* assay should also be able to detect all known haplotypes and be able to detect potential future incursions of *D. vexillum* around Australia. Although the real-time PCR assay is not able to specify which haplotype is detected, this provides confirmation that testing new specimens will not result in a false negative. If required, barcode sequencing could be subsequently carried out to determine the haplotype. The assay represents a powerful tool for routine marine biosecurity diagnostic and management purposes, as it allows for positive identification of *D. perlucidum* or *D. vexillum* to be reported within 24 h from sample reception, allowing for the timely establishment of emergency responses and/or establishment of control strategies. This represents an advantage over the 2- to 3-day turnaround expected from DNA barcoding, and a much-needed faster alternative to the identification provided by the few expert taxonomists currently abroad.

The lack of COI haplotype diversity (with one predominant haplotype) is common in introduced ascidian species worldwide (Turon *et al.* 2003; Rocha *et al.* 2012; Stefaniak *et al.* 2012; Ordóñez *et al.* 2015), and therefore, the method is expected to be useful in detecting *D. perlucidum* at introduced worldwide locations. However, as with many other tropical introduced ascidians in Australia (Kott 2005; Zhan *et al.* 2010; Torkkola *et al.* 2013), the *D. perlucidum* native range and full COI haplotype diversity are unknown (Lambert 2002). Also, despite the

high number of *Didemnum* species reported worldwide (>300 listed in the Ascidiacea World Database) and from Australia alone (Kott 2001, 2005), DNA barcodes were available on the GenBank database for only six species (plus *D. perlucidum* and *D. vexillum*). There is an urgent need to integrate Didemnid taxonomy and molecular identification, so that introduced species can be rapidly and confidently identified worldwide and the specificity of new detection assays can be fully verified.

This study demonstrated the ability of the developed assay to detect *D. perlucidum* eDNA in water samples. It is the first time a real-time PCR assay has been specifically aimed at detecting eDNA of a sessile invertebrate species in the marine environment and, most importantly, in an applied marine biosecurity context. Results of this initial study also suggest that the ability to detect colonial ascidians is largely correlated with their seasonal variation in colony size and larval abundance. At HBH, the strongest detections were obtained in May (Table 3), when visual surveys verified the highest abundance of *D. perlucidum* colonies at all sites inside the HBH sea walls, and which has been previously identified as the end of the *D. perlucidum* reproductive (spawning) peak period at this site (Muñoz *et al.* 2015). In August and December, the assay detections were not always consistent with visual surveys. The apparent absence of *D. perlucidum* colonies at sites with positive eDNA detections in August (Table 3) was not unexpected, and we believe this is mostly due to the significant retraction in the size of the colonies during the winter months (Muñoz *et al.* 2015).

The capacity to detect *D. perlucidum* from water samples can also be variable due to ascidian limited larvae dispersal ability and potential limited eDNA shedding. The lack of detection in December at sites where *D. perlucidum* colonies were observed (Table 3) could likely be explained by the sessile nature of this invertebrate species. Aquatic organisms whose eDNA has been successfully detected from water samples in previous studies have been larger animals (e.g. fish and dolphins in the marine environment) that are constantly moving and shedding tissue and waste (Foote *et al.* 2012; Thomsen *et al.* 2012). It is very possible that small sessile colonial invertebrates like Didemnids shed very little DNA. Larvae, ova and testes could significantly contribute to the overall species eDNA, but these too have a limited patchy presence as they are known to be nonswimming, and unlikely to travel far (Svane & Young 1989). Limited larval dispersal could explain the negative detections from the control site at all times and the weak eDNA detection at the marina entrance occurring in May (Deiner & Altermatt 2014). In and around the marina, *D. perlucidum* was detected at distances greater than 100 m from the closest known colonies during peak abundance, but the strength of the detection, as indicated by the Ct value, decreased with increasing distance from the source. In the Swan River, where currents may be more pronounced, *D. perlucidum* was detected within only 1–2 m of a known colony but was not detected from the surface. The time of sampling, the characteristics of the site (e.g. tides and boat traffic) and the dispersal ability of the target species can all influence the real-time PCR assay eDNA detections on water samples taken from the aquatic environment (Furlan *et al.* 2015). There are also a multitude of biotic and abiotic conditions that affect the degradation of eDNA in the environment which determines how long it is able to persist and how far it may travel (Strickler *et al.* 2015). Most experiments to this point have focused on the degradation rates of eDNA of vertebrates and results have varied considerably (Dejean *et al.* 2011; Barnes *et al.* 2014), so it is unknown how long sessile invertebrate eDNA will persist. The difficulty in controlling these factors is likely to have hindered more applied work in open marine environments.

The real-time PCR assay success in detecting *D. perlucidum* at sites and/or times where it could not be visually detected indicates that marine biosecurity could benefit from incorporating such assays in survey design. The opposite (failure of eDNA detection despite visual confirmation), however, supports the use of the assay on complex environmental samples like water as a complementary method and not a replacement to visual screening methods. In established molecular facilities within biosecurity monitoring agencies where real-time PCR is routinely used (Dias *et al.* 2013), such assays can similarly be applied to the screening of pools of scrapes from pylons and settlement arrays, and plankton samples derived from commercial ports and ballast water. Due to their species-specific nature, they can be mostly useful when targeting a small number of species.

In a not-too-distant future, the establishment of HTS metabarcoding protocols for marine biosecurity should greatly improve the sensitivity and consequent ability for early simultaneous detection of a high number of NIS eDNA from complex marine environmental samples (Pochon *et al.* 2013; Comtet *et al.* 2015). The amount of data generated can also provide an important baseline for monitoring environmental change at anthropogenic impacted sites. At the moment however, such expertise is only provided by a limited number of laboratories.

Using water samples from Hillarys, HTS was initially explored as a pilot study, using MiSeq sequencer (Illumina) Next Generation Sequencing methodology (results not included). While *D. perlucidum* was detected, the preliminary results indicated that further optimization of the primers would be required to allow for the consistent detection of the *Didemnum* species. The limited expertise

coupled with cost and technical implications is likely why, in aquatic environments, conventional PCR and real-time PCR continue to be the monitoring methods of choice in application to complex environmental samples (Ficetola *et al.* 2008; Loh *et al.* 2014) including eDNA detection (Jerde *et al.* 2011; Takahara *et al.* 2013; Deiner & Altermatt 2014; Treguier *et al.* 2014; Spear *et al.* 2015).

Continuous improvement can be made to the sensitivity of all molecular-based methods by evaluating and optimizing the survey technique to be specific to the conditions of the survey area and the dispersal of DNA molecules or larvae for the target species (Furlan *et al.* 2015). The usefulness of the high amount of data obtained from HTS metabarcoding technologies is also, at present, greatly limited by the availability of barcodes in reference databases to assist interpretation (Ratnasingham & Hebert 2007). However, as genetic diversity continues to be described through extremely valuable and most necessary worldwide barcoding initiatives like BoLD, the confidence, application and value of molecular tools such as PCR-based methods and HTS metabarcoding in supporting routine environmental monitoring are expected to increase (Bohmann *et al.* 2014; Thomsen & Willerslev 2015; Zaiko *et al.* 2015).

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References

Armstrong KF, Ball SL (2005) DNA barcodes for biosecurity: invasive species identification. *Philosophical Transactions of the Royal Society B*, **360**, 1813–1823.

Barnes MA, Turner CR, Jerde CL, Renshaw MA, Chadderton WL, Lodge DM (2014) Environmental conditions influence eDNA persistence in aquatic systems. *Environmental Science and Technology*, **48**, 1819–1827.

Bax N, Carlton JT, Mathews-Amos A *et al.* (2001) The control of biological invasions in the world's oceans. *Conservation Biology*, **15**(5), 1234–1246.

Bax N, Williamson A, Aquero M, Gonzalez E, Geeves W (2003) Marine invasive alien species: a threat to global biodiversity. *Marine Policy*, **27** (4), 313–323.

Benson D, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW (2013) GenBank. *Nucleic Acids Research*, **41**, D36–D42.

Bohmann K, Evans A, Gilbert MTP *et al.* (2014) Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in Ecology and Evolution*, **29**(6), 358–367.

Bott NJ, Ophel-Keller KM, Sierp MT *et al.* (2010) Toward routine, DNA-based detection methods for marine pests. *Biotechnology Advances*, **28**, 706–714.

Bridgwood SD, Muñoz J, McDonald JI (2014) Catch me if you can! The story of a colonial ascidian's takeover bid in Western Australia. *BioInvasions Records*, **3**(4), 217–223.

Comtet T, Sandionigi A, Viard F, Casiraghi M (2015) DNA (meta)barcoding of biological invasions: a powerful tool to elucidate invasion processes and help managing aliens. *Biological Invasions*, **17**, 905–922.

Darling JA, Blum MJ (2007) DNA based methods for monitoring invasive species: a review and prospectus. *Biological Invasions*, **9**, 751–765.

Darling JA, Mahon AR (2011) From molecules to management: adopting DNA-based methods for monitoring biological invasions in aquatic environments. *Environmental Research*, **111**, 978–988.

Deagle BE, Bax N, Hewitt CL, Patil JG (2003) Development and evaluation of a PCR-based test for detection of *Asterias* (Echinodermata: Asteroidea) larvae in Australian plankton samples from ballast water. *Marine and Freshwater Research*, **54**, 709–719.

Deiner K, Altermatt F (2014) Transport distance of invertebrate DNA in a natural river. *PLoS ONE*, **9**(2), e88786.

Dejean T, Valentini A, Duparc A *et al.* (2011) Persistence of environmental DNA in freshwater ecosystems. *PLoS ONE*, **6**(8), e23398. doi:10.1371/journal.pone.0023398.

Dias PJ, Fotedar S, Gardner JPA, Snow M (2013) Development of sensitive and specific molecular tools for the efficient detection and discrimination of potentially invasive mussel species of the genus Perna. *Management of Biological Invasions*, **4**(2), 155–165.

Dias PJ, Rocha R, Godwin S *et al.* (2016) Investigating the cryptogenic status of the sea squirt *Didemnum perlucidum* (Tunicata, Ascidiacea) in Australia based on a molecular study of its global distribution. *Aquatic Invasions*, **11**, 239–245.

Ficetola GF, Miaud C, Pompanon F, Taberlet P (2008) Species detection using environmental DNA from water samples. *Biology Letters*, **4**, 423–425.

Foote AD, Thomsen PF, Sveegaard S *et al.* (2012) Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals. *PLoS ONE*, **7**, e41781.

Furlan EM, Gleeson D, Hardy CM, Duncan RP (2015) A framework for estimating the sensitivity of eDNA surveys. *Molecular Ecology Resources*, **16**. doi:10.1111/1755-0998.12483.

Giblot-Ducray D, Bott NJ (2013) Development of a plankton sampling protocol for molecular testing of marine pests. Report prepared for Biosecurity SA. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2013/000281-1. SARDI Research Report Series No. 734. 25 pp.

Gunasekera RM, Patil JG, McEnnulty FR, Bax NJ (2005) Specific amplification of mt-COI gene of the invasive gastropod *Maoricolpus roseus* in planktonic samples reveals a free-living larval life-history stage. *Marine and Freshwater Research*, **56**, 901–912.

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London Series B- Biological Sciences*, **270**, 313–321.

Hulme PE (2006) Beyond control: wider implications for the management of biological invasions. *Journal of Applied Ecology*, **43**, 835–847. doi:10.1111/j.1365-2664.2006.01227.

Jerde CL, Mahon AR, Chadderton WL, Lodge DM (2011) "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters*, **4**, 150–157.

Kott P (2001) The Australian Ascidiacea Pt 4, Didemnidae. *Memoirs of the Queensland Museum*, **47**(1), 1–410.

Kott P (2005) New and little-known species of Didemnidae (Ascidiacea, Tunicata) from Australia (Part 3). *Journal of Natural History*, **39**(26), 2409–2479.

Lambert G (2002) Nonindigenous ascidians in tropical waters. *Pacific Science*, **56**(3), 291–298.

Loh WKW, Bond P, Ashton KJ, Roberts DT, Tibbetts IR (2014) DNA barcoding of freshwater fishes and the development of a quantitative qPCR assay for the species-specific detection and quantification of fish larvae from plankton samples. *Journal of Fish Biology*, **85**, 307–328.

López-Legentil S, Legentil ML, Erwin PM, Turon X (2015) Harbor networks as introduction gateways: contrasting distribution patterns of native and introduced ascidians. *Biological Invasions*, **17**, 1623–1638.

Muñoz J, McDonald J (2014) Potential eradication and control methods for the management of the ascidian *Didemnum perlucidum* in Western Australia. Fisheries Research Report No 252. Department of Fisheries, Western Australia. 40 pp.

Muñoz J, Page M, McDonald JI, Bridgwood SD (2015) Aspects of the growth and reproductive ecology of the introduced ascidian *Didemnum perlucidum* (Monniot, 1983) in Western Australia. *Aquatic Invasions*, **10**(3), 265–274.

Ordóñez V, Pascual M, Fernández-Tejedor M, Pineda MC, Tagliapietra D, Turon X (2015) Ongoing expansion of the worldwide invader *Didemnum vexillum* (Ascidiacea) in the Mediterranean Sea: high plasticity of its biological cycle promotes establishment in warm waters. *Biological Invasions*, **17**, 2075–2085.

Pagad S, Hayes K, Katsanevakis S, Costello MJ (2015) World Register of Introduced Marine Species (WRIMS). http://www.marinespecies.org/introduced.

Pfaffl MW (2004) Quantification strategies in real-time PCR. In: *A-Z of Quantitative PCR*(ed Bustin S. A.), Chapter 3, 25 pp. International University Line, La Jolla.

Pimentel D, Lach L, Zuniga R, Morrison D (2000) Environmental and economic cost of nonindigenous species in the United States. *BioScience*, **50**(1), 53–65.

Pochon X, Bott NJ, Smith KF, Wood SA (2013) Evaluating detection limits of Next Generation sequencing for the surveillance and monitoring of international marine pests. *PlosOne*, **8**(9), e73935.

Ratnasingham S, Hebert PDN (2007) BOLD: the barcode of life data system (www.barcodinglife.org). *Molecular Ecology Notes*, **7**, 355–364.

Rilov G, Crooks JA (eds.) (2009) *Biological Invasions in Marine Ecosystems: Ecological, Management and Geographic Perspectives*. Springer-Verlag, Berlin.

Rocha RM, Kremer LP, Fehlauer-Ale KH (2012) Lack of COI variation for *Clavelina oblonga* (Tunicata, Ascidiacea) in Brazil: Evidence for its human-mediated transportation? *Aquatic Invasions*, **7**(3), 419–424.

Simberloff D (2001) Eradication of island invasives: practical actions and results achieved. *Trends Ecology and Evolution*, **16**, 273–274.

Simpson TJS, Wernberg T, McDonald J (2016) Distribution of the invasive ascidian *Didemnum perlucidum* in an urban estuary and impact on seagrass communities. *PLoS ONE*, **11**, e0154201.

Smale DA, Childs S (2012) The occurrence of a widespread marine invader, *Didemnum perlucidum* (Tunicata, Ascidiacea) in Western Australia. *Biological Invasions*, **14**, 1325–1330.

Smith KF, Wood SA, Mountfort DO, Cary SC (2012a) Development of a real time PCR assay for detection of the invasive clam, *Corbula amurensis* in environmental samples. *Journal of Experimental Marine Biology and Ecology*, **412**, 52–57.

Smith KF, Stefaniak L, Saito Y, Gemmill CEC, Cary SC, Fidler AE (2012b) Increased inter-colony fusion rates are associated with reduced COI haplotype diversity in an invasive colonial ascidian *Didemnum vexillum*. *PLoS ONE*, **7**(1), e30473.

Spear SF, Groves JD, Williams LA, Waits LP (2015) Using environmental DNA methods to improve detectability in a hellbender (*Cryptobranchus alleganiensis*) monitoring program. *Biological Conservation*, **183**, 38–45.

Stefaniak L, Lambert G, Gittenberger A, Zhang H, Lin S, Whitlatch RB (2009) Genetic conspecificity of the worldwide populations of *Didemnum vexillum* (Kott, 2002). *Aquatic Invasions*, **4**, 29–44.

Stefaniak L, Zhang H, Gittenberger A, Smith K, Holsinger Lin S, Whitlatch RB (2012) Determining the native region of the putatively invasive ascidian *Didemnum vexillum* Kott, 2002. *Journal of Experimental Marine Biology and Ecology*, **422–423**, 64–71.

Stewart-Clark SE, Siah A, Greenwood S, Davidson J, Berthe FCJ (2009) Development of 18S rDNA and COI gene primers for the identification of invasive tunicate species in water samples. *Aquatic Invasions*, **4**(4), 575–580.

Stewart-Clark SE, Davidson J, Greenwood SJ (2013) Optimization and validation of molecular assays for invasive tunicate monitoring in environmental water samples. *Aquatic Science and Technology*, **1**, 143–165.

Strickler KM, Fremier AK, Goldberg CS (2015) Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. *Biological Conservation*, **183**, 85–92.

Svane IV, Young CM (1989) The ecology and behavior of ascidian larvae. In: *Oceanography and Marine Biology—An Annual Review*, **27**, 45–90.

Takahara T, Minamoto T, Doi H (2013) Using environmental DNA to estimate the distribution of an invasive fish species in ponds. *PLoS ONE*, **8**(2), e56584.

Thomsen PF, Willerslev E (2015) Environmental DNA—an emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, **183**, 4–18.

Thomsen PF, Kielgast J, Iversen LL, Moller PR, Rasmussen M, Willerslev E (2012) Detection of a diverse marine fish fauna using environmental DNA from seawater samples. *PLoS ONE*, **7**(8), e41732.

Torkkola J, Riginos C, Liggins L (2013) Regional patterns of mtDNA diversity in *Styela plicata*, an invasive ascidian, from Australian and New Zealand marinas. *Marine and Freshwater Research*, **64**, 139–145.

Treguier A, Paillisson J-M, Dejean T, Valentini A, Schlaepfer MA, Roussel J-M (2014) Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. *Journal of Applied Ecology*, **51**(4), 871–879. doi:10.1111/1365-2664.12262.

Turon X, Tarjuelo I, Duran S, Pasucal M (2003) Characterising invasion processes with genetic data: an Atlantic clade of *Clavelina lepadiformis* (Ascidiacea) introduced into Mediterranean harbours. *Hydrobiologia*, **503**, 29–35.

Valasek MA, Repa JJ (2005) The power of real-time PCR. *Advances in Physiology Education*, **29**, 151–159. doi:10.1152/advan.00019.

Wood SA, Smith KF, Banks JC *et al*. (2013) Molecular genetic tools for environmental monitoring of New Zealand's aquatic habitats, past, present and the future. *New Zealand Journal of Marine and Freshwater Research*, **47**, 90–119.

Zaiko A, Samuiloviene A, Ardura A, Garcia-Vazquez E (2015) Metabarcoding approach for nonindigenous species surveillance in marine coastal waters. *Marine Pollution Bulletin*, **100**, 53–59.

Zhan A, MacIsaac HJ, Cristescu ME (2010) Invasion genetics of the *Ciona intestinalis* species complex: from regional endemism to global homogeneity. *Molecular Ecology*, **19**, 4678–4690.

The experimental design of this study was developed by Tiffany Simpson, Joana Dias and Julieta Muñoz. The authors also shared the tasks of collecting and filtering all of the water samples as well as extracting the DNA from both tissue and water. Joana Dias developed the primer sets for *Didemnum perlucidum* and *Didemnum vexillum*, and T.J.S.S. completed all qPCR optimization and screening of water sample DNA with the real-time PCR assay. Aliquots of DNA were given to Tina Berry from Curtin University to trial the use of the *D. perlucidum* primers with next-generation sequencing techniques. As the lead author, Tiffany Simpson compiled all of the results and produced the first and final drafts of the manuscript. Input and editing was provided by the coauthors Joana Dias, Mike Snow and Tina Berry.

Data accessibility

DNA sequences: GenBank accessions JQ731735, JQ731740, JQ731736-9, JX846617, KF309576, JQ692626-8, EU742661, JQ780669, JQ780673, JQ780675, JQ780687 and JQ780688; and BoLD accession OZIMP002-15.

Final DNA sequence assembly and real-time PCR results have been uploaded as online supplementary material.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Real-time PCR Ct values of Didemnum perlucidum from eDNA, larvae and dilution series.

Table S2 Alignment of Didemnum sequences used for the design of Taqman primers and probes.

SCIENTIFIC REPORTS

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Ecosystem biomonitoring with eDNA: metabarcoding across the tree of life in a tropical marine environment

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Effective marine management requires comprehensive data on the status of marine biodiversity. However, efficient methods that can document biodiversity in our oceans are currently lacking. Environmental DNA (eDNA) sourced from seawater offers a new avenue for investigating the biota in marine ecosystems. Here, we investigated the potential of eDNA to inform on the breadth of biodiversity present in a tropical marine environment. Directly sequencing eDNA from seawater using a shotgun approach resulted in only 0.34% of 22.3 million reads assigning to eukaryotes, highlighting the inefficiency of this method for assessing eukaryotic diversity. In contrast, using 'tree of life' (ToL) metabarcoding and 20-fold fewer sequencing reads, we could detect 287 families across the major divisions of eukaryotes. Our data also show that the best performing 'universal' PCR assay recovered only 44% of the eukaryotes identified across all assays, highlighting the need for multiple metabarcoding assays to catalogue biodiversity. Lastly, focusing on the fish genus *Lethrinus*, we recovered intra- and inter-specific haplotypes from seawater samples, illustrating that eDNA can be used to explore diversity beyond taxon identifications. Given the sensitivity and low cost of eDNA metabarcoding we advocate this approach be rapidly integrated into biomonitoring programs.

Marine ecosystems are under increasing pressure from a variety of anthropogenic stressors, including climate change and fishing activities[1–4]. Habitat degradation, overexploited fisheries, altered food web dynamics and shifts in community composition highlights the need for effective management of the marine biome in order to preserve and manage ocean resources sustainably into the future[4,5]. A fundamental tool for providing data to support management of the marine environment is biomonitoring. By targeting indicator species or repeating surveys of specific sites, regular monitoring of the biota allows impacts affecting the abundance and/or alpha and beta diversity to be measured[6]. However, current approaches to surveying conspicuous faunal elements (e.g. baited remote underwater video, transects, or diver tows) are dependent on suitable field conditions, a particular set of skills, and are limited to the narrow portion of biodiversity recorded. Indeed, community assemblage surveys are often restricted to a single taxonomic group such as fishes or corals, or even a subset of those groups[7,8]. Considering the growing evidence that total biodiversity promotes healthy ecosystem functions and that sustaining biodiversity represents a practical framework for ecosystem-based management (EBM), there is a compelling need for more comprehensive approaches to monitoring marine biota[9–13].

Recent advances in genomic technologies afford new opportunities across a broad range of applications in environmental science[14–18]. One such application lies in the discovery that organisms leave traces of their DNA in the environment, including seawater, and when extracted, is collectively referred to as eDNA[19–21]. Here, we refer to eDNA as all genetic material that is obtained directly from the environment as in Taberlet *et al.*[21], which depending on sampling methodology, constitutes DNA from whole organisms (i.e. prokaryotes and microscopic

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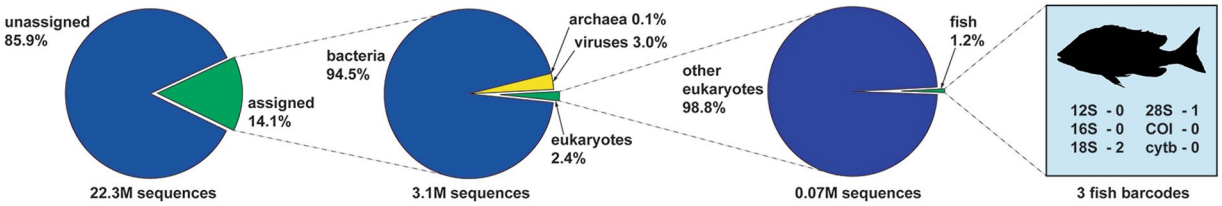

Figure 1. Assignment of sequences recovered from the shotgun library of eDNA collected from Coral Bay in west Australia. Pie chart segments represent the percentage of sequences that were assigned to taxa using the software MEGAN 5.11.3. Sequences that were assigned to fish were further mined for commonly used DNA barcodes (12S, 16S, 18S, 28S, COI, and cyt*b*); the number of fish barcodes identified in the dataset is displayed in the box insert.

eukaryotes), cellular material (i.e. blood, mucous, tissue, faeces, etc.), and that which is released from the cytoplasm as free nucleic acids. The potential utility of eDNA to advance the scientific process is broad, ranging from questions related to species detection, biodiversity assessments, population genetics, reconstruction of past flora and fauna and the detection of invasive marine species[22–26]. To date however, the capacity of eDNA to inform on ecosystem-wide patterns of biodiversity (i.e. from prokaryotes to higher-order eukaryotes), in a marine environment, remains largely unexplored.

A number of methodologies can be employed in the analysis of eDNA. One approach is environmental shotgun sequencing (ESS), which randomly sequences fragmented DNA directly from an environmental sample[27]. As ESS does not enrich target DNA, the cost associated with sequencing the entire DNA complement present in a sample is prohibitive[28]. Furthermore, ESS of genetic material recovered from seawater has mostly been applied to the study of prokaryotes[29,30] and picoeukaryotes[31], and so its utility for characterising eDNA originating from eukaryotes, particularly macroeukaryotes, needs further investigation.

To overcome the cost and quantity of DNA that needs to be analysed when using an ESS approach, PCR amplification of target genes (and taxa) on bulk DNA extracts from the environment can be combined with next-generation sequencing (NGS) to provide high-throughput information on the species present, a technique commonly referred to as DNA metabarcoding[32]. While this approach has proven useful in detecting a high diversity of species from a variety of environmental samples (e.g. soil and water), the influence of PCR-bias on taxonomic recovery and limited correlations to biomass of target species have led researchers to explore the utility of other PCR-free methods[33–37]. For example, gene-enrichment approaches employ synthetic probes that bind and purify target DNA of interest followed by NGS[38]. While gene-enrichment is a powerful method in detecting taxa of interest from bulk samples (e.g. synthetic mixtures of macroinvertebrates[39]), its application across the remaining taxa is currently prohibitive due to the high cost of probes that would capture target genes from all other organisms present in the environment.

The capacity of eDNA to inform on eukaryotic diversity from aquatic environments was described in 2008[20], and yet there is no single study that we are aware of that has explored the utility of eDNA methodologies to assess marine biota at a holistic ecosystem level (i.e. across the tree of life). Accordingly, before temporal and spatial surveys using eDNA can be implemented, the scope and resolution at which metabarcoding can inform on biodiversity in the ocean needs to be validated[17]. While it has been shown previously that eDNA methodologies are superior to conventional surveying methods in detecting species within aquatic environments through rigorous *in silico* and *in vitro* (PCR) analysis of primer sets designed for specific taxa (e.g. amphibians and fish[40]), the capacity for the analysis of eDNA from seawater to broadly describe total biodiversity in any given sample requires further investigation. Therefore, in this study, we focused on a single tropical coral reef site to explore the ability of eDNA to audit marine biota across the entire tree of life. We chose to sample seawater from Coral Bay in west Australia as it resides within the World Heritage site of Ningaloo Reef, which is renowned for its rich marine biodiversity and enigmatic megafauna, is one of the world's longest fringing coral reefs, and is therefore of high conservation importance[41]. Rather than perform rigorous tests on the detection of specific groups of taxa using eDNA[34,40,42], we instead focused on assessing the broad potential of eDNA for auditing marine taxa. To appraise the efficacy of different methodologies for the study of eDNA in the ocean, we analysed over 23 million sequences originating from 9 L of filtered seawater and compared the diversity of taxa detected at Coral Bay using ESS and metabarcoding. We further investigated the potential of eDNA to inform on intra-species diversity using mitochondrial haplotype data, with the intention of developing approaches for the use of eDNA in measuring population diversity for a commercially targeted fish genus in Northwestern Australia[43].

Results and Discussion

Shotgun sequencing of eDNA from seawater. We built a Nextera XT library from the DNA extracted from 9 L of seawater collected at Coral Bay, generating 22.3 million reads (single end 151 bp fragments) on an Ilumina NextSeq platform using an ESS approach. Of the 22.3 million DNA sequences in the shotgun library that passed quality filtering, only 14.1% could be assigned to taxa using Blastn (Fig. 1), with the highest proportion of hits assigning to bacteria (94.5%), followed by viruses (3.0%), eukaryotes (2.4%) and archaea (0.1%). Of the sequences assigning to eukaryotes, ~5000 reads matched with commonly used DNA barcodes (12S, 16S, 18S, 28S, COI, cyt*b*). The overall low level of taxonomic assignment and the proportionally high number of hits to prokaryotes compared to eukaryotes using the ESS data is not surprising when considering the bias of the existing reference databases and relative abundance of these taxonomic groups in seawater. There are only 3339 eukaryotic,

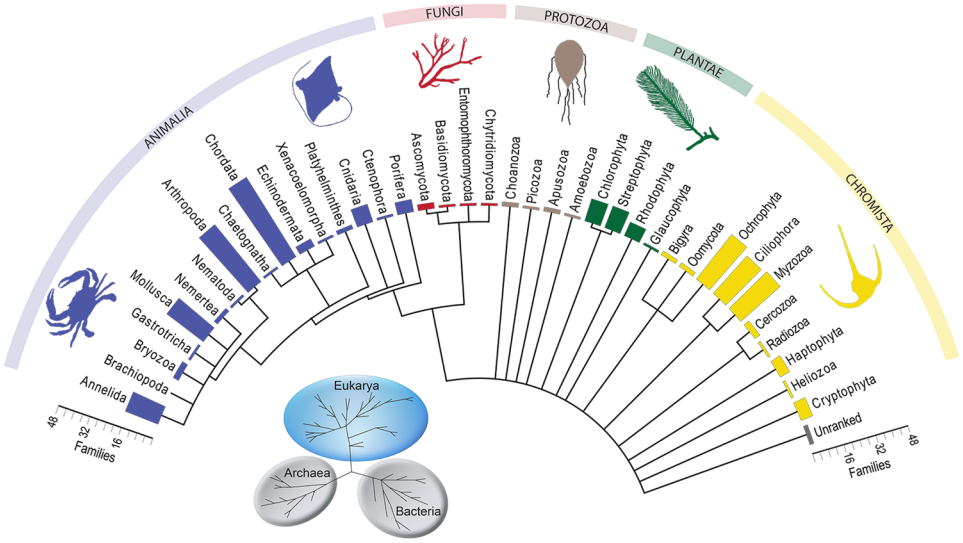

Figure 2. Taxonomic phylogram of eukaryotic diversity at Coral Bay in west Australia derived from ToL-metabarcoding. Bar graphs indicate the number of families in each phyla characterised at Coral Bay, and are coloured according to kingdom.

72311 prokaryotic and 5646 viral genomes currently on GenBank (accessed 10 August 2016), many of which are sourced from terrestrial organisms. Further, while there is genetic information on more taxa through a growing database of commonly used barcode genes, these regions only represent a small fraction of an organism's genome. Therefore, considering that the library preparation of eDNA for ESS includes all of the genetic material (i.e. entire genomes) from potentially all of the taxa that were present at Coral Bay, the likelihood of recovering DNA fragments mapping to barcode regions that have previously been sequenced and are publically available is low. The frequency of eukaryotic hits in the ESS data also reflects the relatively low abundance of micro-eukaryotes in our oceans, as there are estimated to be approximately 10000 viruses, 1000 bacteria, and 20 micro-eukaryotes in 1 mL of seawater[44]. Therefore, due to the higher number of bacteria that are sampled compared to micro-eukaryotes, and the higher number of prokaryotic versus eukaryotic genomes sequenced (72311 to 3339, respectively), the chance of mapping ESS data to bacterial genes is far greater. Finally, the recovery of eDNA from macroscopic eukaryotes (e.g. metazoans) is restricted to DNA contained in biological secretions, larvae or decaying cells, which is in much lower concentration than eDNA derived from prokaryotes and micro-eukaryotes[45].

To critically evaluate the capacity of the ESS data to inform on the non-microbial fraction of the eDNA library, we focused on recovering fish DNA fragments rather than characterise the total diversity present in the library given their well-described taxonomy relative to other groups. The initial Blastn search using NCBI assigned 1.2% of the eukaryotic sequences to fish (class Actinopterygii, Chondrichthyes and Cyclostomata; ~875 reads of the original 22.3 M reads − 0.00004%), which is a poor relative representation. The majority of these fish reads mapped to genome assemblies, mRNA or phylogenetic informative genes. With regards to assigning taxonomic identity, the most valuable reads are instead those that map on to commonly used reference barcodes[46]. Searching the 22.3 million reads, only three sequences that are commonly used as DNA barcodes mapped to fish (Fig. 1); two 18S rDNA sequences, which could only resolve to the class Actinopterygii (matching six fishes with equal similarity; Bit Score = 248.348), and one 28S rDNA fragment with similar low-resolving power (Bit Score = 161.786). The low number of reads and lack of resolving power for fish in the ESS dataset showcases the limited ability of ESS data to inform on eukaryotic diversity.

Therefore, while shotgun sequencing using NGS platforms may represent the most unbiased way to explore eDNA from seawater, and has proven useful for the study of bacteria[47], we demonstrate that the application of ESS for eukaryotes is currently not feasible and lacks resolution compared to metabarcoding (see below). Even for abundant and well-characterised marine taxa such as fish, NGS and ESS was unable to 'cut through' the microbial biomass that overwhelms the data that we recovered. While enrichment of target species through capture-probes is possible[39], it is currently cost-prohibitive for use in routine monitoring of marine ecosystems due to the unwieldy number of libraries and probes that would be required for coverage across all taxa.

Metabarcoding of eDNA from seawater. The lack of non-microbial taxa detected from the shotgun sequencing data led us to explore the potential of ToL-metabarcoding, which we define as the use of multiple metabarcoding assays to survey a wide array of biotic diversity. Using ten different metabarcoding assays, a total of 1.2 million amplicon reads (that passed quality filtering) were used to generate a multi-taxon eDNA snapshot of the marine biodiversity at Coral Bay, 20-fold less sequencing effort than that used for ESS. Compared to the 14.1% of the ESS data that could be assigned to taxa, 79.7% of the metabarcoding sequence data could be assigned to taxa at Coral Bay (Supplementary Datas 1 and 2). The metabarcoding data was assigned to 434 eukaryotic taxa: 38 phyla, 88 classes, 186 orders and 287 families (Fig. 2; Table 1; Supplementary Data 3). Likewise for prokaryotes,

Kingdom	Phylum	Class	Order	Family	Genus	Species
Animalia	Annelida	2	7	17	16	
	Arthropoda	3	12	35	38	8
	Brachiopoda	1				
	Bryozoa	1	1	3		
	Chaetognatha		1	1	1	
	Chordata	5	17	45	73	36
	Cnidaria	2	5	9	7	
	Ctenophore	1	1	1	1	
	Echinodermata	3	2	3	4	2
	Gastrotricha		1	1		
	Mollusca	4	15	22	19	5
	Nematoda	1	1	1		
	Nemertea	2	1	2	2	
	Platyhelminthes	4	3	2		
	Porifera	3	6	6	6	
	Xenacoelomorpha	1		1		
Chromista	Bigyra	1	2	2	3	
	Cercozoa	4	4	3	4	
	Ciliophora	7	15	24		
	Cryptophyta	3	3	6	6	
	Haptophyta	4	5	5	4	1
	Heliozoa	1	1	1	1	
	Myzozoa	3	10	25	36	2
	Ochrophyta	9	31	27		
	Oomycota	1	3	2		
	Radiozoa	2	2	1	1	
Fungi	Ascomycota	3	4	3		
	Basidiomycota	2	2	1	1	
	Chytridiomycota	1	1	1	1	
	Entomophthoromycota	1	1	1	1	
Plantae	Chlorophyta	7	10	11	17	
	Glaucophyta	1		1	1	
	Rhodophyta	1	5	7	3	
	Streptophyta	1	11	11	2	
Protozoa	Amoebozoa		1	1	1	
	Apusozoa			2	3	
	Choanozoa	2	1	2	3	
	Picozoa	1	1	1		
Total	**38**	**88**	**186**	**287**	**255**	**54**

Table 1. The number of eukaryotic taxa identified within each taxonomic rank at Coral Bay in west Australia via ToL-metabarcoding.

445 Operational Taxonomic Units (OTUs) from 14 phyla, 28 classes, 61 orders and 96 families were detected (Fig. 3; Supplementary Data 3). By using a suite of metabarcoding assays that target different organisms, all the major taxonomic lineages including the Animalia, Fungi, Protozoa, Plantae, Chromista, Bacteria, and Archaea were detected. For example, three classes of vertebrates were recovered from metabarcoding; Actinopterygii (ray-finned fishes), Chondrichthyes (cartilaginous fishes) and Mammalia. The majority of vertebrate diversity was within the class Actinopterygii (41 families), whereas whiptail stingrays (Dasyatidae) and eagle rays (Myliobatidae), as well as dolphins (Delphinidae), made up the families detected in the class Chondrichthyes and Mammalia, respectively. Thirty classes of invertebrates from 16 phyla, including arthropods and benthic organisms such as cnidarians (which includes corals), sponges and bivalves, were also detected from seawater samples. Interestingly, ten classes (31 families) of plants from four phyla were detected, including some of terrestrial origin, which were likely encountered via dispersal of pollen by wind and freshwater[48]. Micro-eukaryotes, such as fungi and phytoplankton, as well as prokaryotes, including dominant bacterioplankton such as SAR11, SAR 86, SAR116 and OM43[49], were also present in the samples. Rather than critically evaluate the efficiency of each PCR assay used, what this study showcases is the capacity to audit the entire spectrum of taxonomic diversity present in a tropical marine environment using DNA samples extracted from seawater. A similar approach using six PCR assays on eDNA extracted from soil samples detected terrestrial prokaryotes and eukaroytes[50], and recent studies on eDNA extracted from seawater and settlement plates detected a wide range of eukaryotes[51–54]. Collectively,

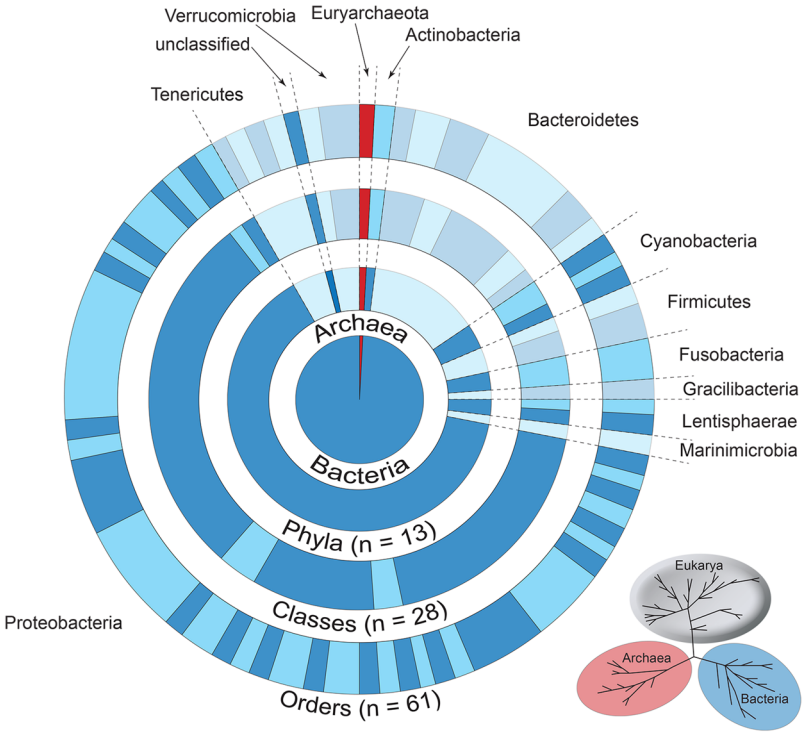

Figure 3. Hierarchical pie chart of prokaryotic diversity at Coral Bay in west Australia derived from ToL-metabarcoding. The inner pie chart represents the relative proportion of families identified in bacteria (blue) and archaea (red). Each segmented circle illustrates the number of taxa (phyla, classes and orders) characterised for both bacteria and archaea, and is scaled according to the number of families within each rank. Dotted lines partition the number of taxa within each phylum, which are named around the circumference of the chart.

these studies indicate that eDNA methodologies can be used on substrates from a variety of environments to assess a broad range of taxa. With further methodological development, ToL-metabarcoding provides compelling evidence for its inclusion into a biomonitoring 'toolkit' for marine environments.

When comparing ESS to ToL-metabarcoding, it is clear that the latter method is superior in detecting fish taxa using eDNA. While only three barcode sequences could be assigned to the well-characterised Actinopterygii using ESS (and could not resolve to lower taxonomic levels), 69 Actinopterygii taxa were resolved using ToL-metabarcoding, including 33 to the species level. Further, the application of specific primers for the detection of fishes on eDNA collected from water indicates advantages over traditional survey methods. Analysis of eDNA recovered higher numbers of fish taxa in both marine and freshwater systems compared to traditional surveying methods[55,56], and when comparing eDNA surveys to known fish diversity present in aquaria (e.g. Okinawa Churaumi Aquarium, 180 fish species), Miya *et al.*[34] were able to identify >90% of taxa from their 12S rRNA sequences. In our data set, we detected small "bait fishes" from the genera *Atherinomorus*, *Engraulis*, *Hypoatherina*, *Hyporhamphus*, *Sardinella*, *Spratelloides* and *Strongylura*. While these genera are sometimes seen and recorded by other techniques such as Underwater Visual Census (UVC) by SCUBA divers or Baited Remote Underwater Video (BRUV) systems, it is extremely challenging to identify fish to species-level even if the family can be determined visually[7]. We also detected a number of cryptobenthic fishes with eDNA (e.g. Families Blenniidae, Gobiidae and Pseudochromidae) and fishes that are mostly active at night (e.g. families Apogonidae, Gerreidae and Holocentridae), which are usually not sampled adequately by other techniques[7,57,58]. Several species from families targeted by recreational and commercial fishers (i.e. Carangidae, Lethrinidae, Lutjanidae, Mugilidae, Epinephelidae) were also recovered from the metabarcoding data. As eDNA methodologies are non-invasive and show high sensitivity and detection capabilities, the application of metabarcoding eDNA for surveying fish in combination with assays that target other taxa are likely to be adopted in future monitoring programs.

The importance and utility of multiple metabarcoding assays. Our data advocates strongly for the use of ToL-metabarcoding as opposed to relying on a single 'universal' PCR assay to audit biota in the marine environment. The universal PCR assay based on the (commonly employed) 18S rDNA V4 region detected 191 taxa, which represents only 44% of the total number of taxa characterised from all PCR assays combined ($N = 434$; Fig. 4). Further, when normalising for sequencing depth, the trend in the number of taxa resolved for each assay at each taxonomic rank is consistent to that depicted in Fig. 4 (Supplementary Data 4), and there is a significant difference in the number of taxa identified between assays at each taxonomic rank (Kruskal-Wallis rank sum test, $p < 0.05$). Our study indicated that the 18S universal assays detected the greatest number of taxa followed by the COI assay, which is consistent with that reported by Kelly *et al.*[51]. While *in silico* analysis and testing of primer

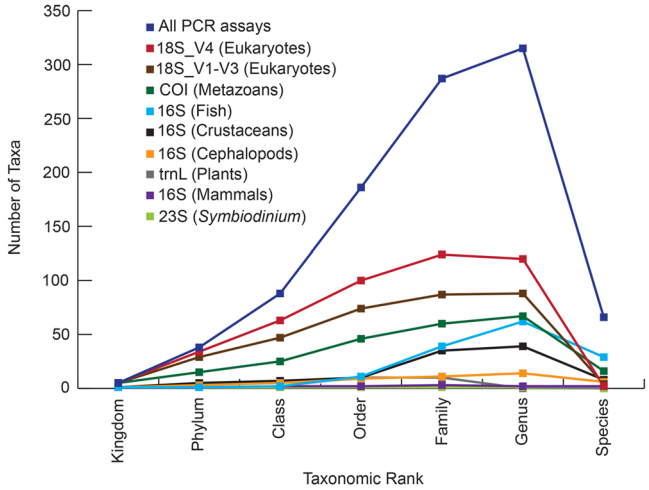

Figure 4. Line graph representing the number of eukaryotic taxa recorded at Coral Bay in west Australia using eDNA. Coloured lines indicate the number of taxa identified for each taxonomic rank for the nine PCR assays that target eukaryotes.

Primer Set	Order	Family	Genus	Species	OTUs
18S_V4F	4	2			16
18S_VR					
18S_1 F	1				2
18S_400 R					
m1COIintF	6	12	15	9	16
jgHCO2198					
16Smam1	1	2	2	2	2
16Smam2					
16SF/D	10	38	61	29	143
16S2R-degenerate					
Multiple Hits	7	12	14	6	

Table 2. The number of fish taxa and OTUs (class Actinopterygii) identified at Coral Bay in west Australia across PCR assays used in the study. The row 'Multiple Hits' refers to the number of taxa identified with more than one PCR assay. OTUs were generated using a 98% similarity cut-off.

sets on synthetic blends is preferred when assessing the efficiency of PCR assays to detect taxa of choice[35], it is unrealistic when the goal is to characterise all organisms present in an ecosystem. This challenge is particularly relevant within regions of unknown biodiversity with poor reference barcodes. However, it is nevertheless clear, that using a suite of universal PCR assays in combination with specific assays that achieve taxonomic saturation (Supplementary Data 5), a greater genetic diversity of taxa is uncovered.

There are a number of challenges that arise with interpreting data from single assays including primer bias, gene copy number, PCR or sequencing artefacts and/or contamination[35,59,60]. The application of numerous assays can overcome many of these issues through multiple detection hits for specific taxa and/or detecting a greater range of species. To showcase the importance of gene and primer choice, Table 2 shows the number of taxa within class Actinopterygii that were identified across all PCR assays employed in the study. Firstly, it can be seen that if a specific group of taxa is important for assessing diversity, in this case the Actinopterygii, taxon-specific assays (versus universal assays) reveal more taxa and much higher levels of diversity. For example, for all levels of taxonomy, the fish 16S assay detected more fish (61 genera) than any other primer set, as well as more genetic diversity as measured by the number of OTUs (Table 2). This highlights the fact that 'universal' assays, while excellent for providing a snapshot of biodiversity, are not able to adequately capture the depth of diversity within specific groups of taxa. This outcome is also influenced by the fact that nuclear rRNA genes (i.e. 18S) typically used as universal assays provide lower taxonomic resolution than mitochondrial rRNA genes (i.e. 16S)[61]. Further, while it would seem ideal to design a fish-specific assay based on the COI gene, which is the barcode of choice for vouchered fish specimens, the primer binding regions are more variable and therefore do not allow for the same specificity as the 16S and 12S regions of the mitochondrial genome[34,61,62]. Secondly, the use of multiple PCR assays can provide higher levels of confidence in the organisms detected when multiple hits to the same group are achieved (e.g. 14 shared fish genera across PCR assays; Table 2). If interested in specific taxa, like fish, then the application of multiple fish specific primers, like the 16S assay employed here in combination with the 12S assay used in Miya *et al.*[34], would be preferred. A second example from our dataset is the recovery and characterisation

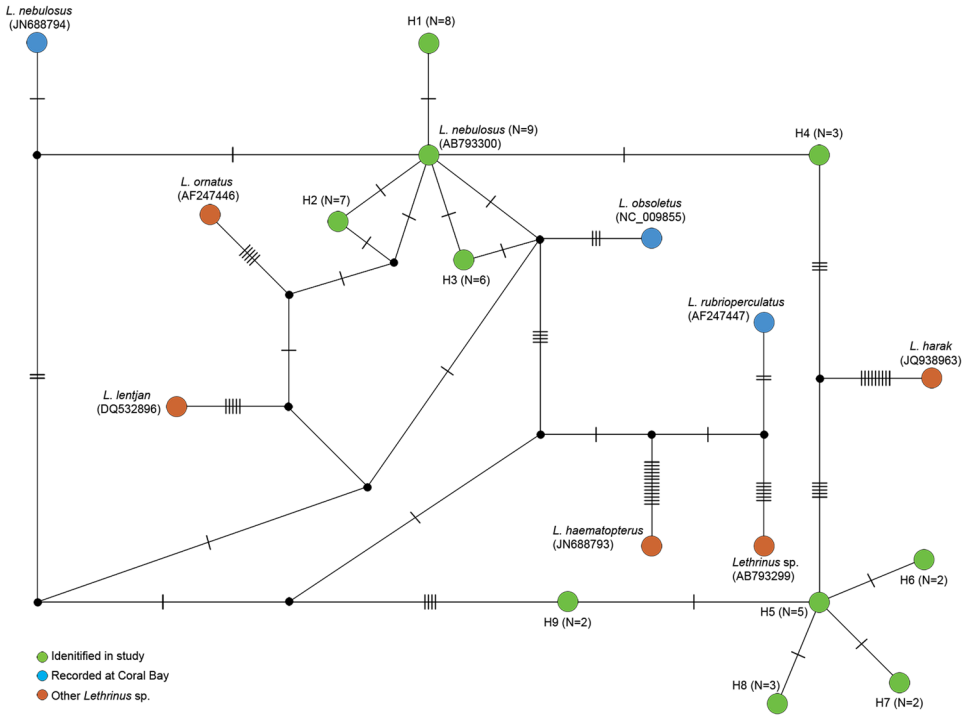

Figure 5. Network of *Lethrinus* 16S rDNA haplotypes. Green circles represent haplotypes identified at Coral Bay in west Australia in this study using eDNA, blue circles represent *Lethrinus* haplotypes obtained from NCBI for additional species recorded at Coral Bay, and red circles represent all other *Lethrinus* haplotypes available from NCBI. Numerals in brackets indicate the number of samples the haplotype was detected in (out of a total of 9). Genbank accession numbers are also indicated in brackets.

of diversity within a single genus. Dinoflagellates belonging to the genus *Symbiodinium* are important photosynthetic symbionts that associate with a wide range of marine invertebrates, including corals, and represent the most abundant phytoplankton in tropical waters[63]. Indeed, a high diversity of taxa within this single genus is important for understanding host associations between plants and animals in our oceans[64]. While our universal assay (18S V4) recovered *Symbiodinium*, the application of a genus-specific assay (cp23S) further resolved seven of the nine major sub-generic phylogenetic lineages (clades) within the genus[65] (Supplementary Data 3), which represents the cornerstone of investigating the potential for corals to adapt to climate change[63,66]. Taken together, these data demonstrate that multiple PCR assays with different target spectrums will collectively provide better recovery of taxa. As each barcode has advantages and disadvantages related to its resolution, taxonomic specificity and availability of reference sequences, the assay design needs to be study specific[61].

Exploring haplotype diversity with eDNA. OTUs are commonly used to assess genetic diversity in a taxonomic independent approach that is free from the constraints of incomplete taxonomic frameworks and reference DNA databases[67,68]. OTU-based approaches are especially useful when comparing complex metabarcoding data from different locations and/or collection times, often via multivariate methods. Here we also investigated the potential of eDNA to inform on intra- and inter-species haplotype diversity beyond the OTU approach, and selected the fish genus *Lethrinus* as a model to explore this given the importance of species in this genus in recreational and commercial fisheries[43]. An error rate of 1.79% ± 1.59 was calculated for fish 16S amplicons (see methods), and was subsequently used to screen out low-frequency sequences from the metabarcoding data recovered from Coral Bay that assigned to *Lethrinus*. This approach is comparable to a recent study investigating whale shark population genetic diversity inferred from eDNA using a frequency cut-off of 1.3%[26]. After screening out low abundance sequences, and those not identified across more than one DNA sample, we detected ten reproducible *Lethrinus* haplotypes at Coral Bay (Fig. 5) that are unlikely to represent sequencing artefacts. One haplotype that we detected was identical to a reference barcode for *L. nebulosus* (GenBank Accession Number: AB793300), a species of *Lethrinus* present at Coral Bay based on the Atlas of Living Australia and complimentary observational surveys[69]. In addition, we detected four haplotypes that were 1 bp different to *L. nebulosus*. Considering that *L. nebulosus* has another reference haplotype 2 bp different (GenBank Accession Number: JN688794), we consider all five of these sequences to represent intra-species diversity for *L. nebulosus* at Coral Bay. A further five haplotypes that clustered together but were separate from other *Lethrinus* sequences in the network were identified in the sequence data. The Lethrinid species that these correlate to, however, is unknown, as there are no 16S reference barcodes from multiple species of *Lethrinus* known to occur in Coral Bay (e.g. *L. atkinsoni*, *L. genivittatus*, *L. laticaudis*, *L. olivaceus*, *L. variegatus*). That said, our haplotype data for *Lethrinus* showcases the capacity to extract and explore a particular species or genus within a more complex dataset (i.e. from all fish species that

are co-amplified in a metabarcoding assay), as opposed to extracting haplotypes from a species-specific dataset, which was done for the whale shark study[26]. Collectively, these analyses demonstrate that when metabarcoding data is properly filtered, eDNA has the ability to extend beyond taxa lists and provide information on the genetic diversity of species across both time and space.

Caveats and future directions. Our data show that ToL-metabarcoding can significantly advance the capacity to monitor tropical environments and implement EBM, but this approach, like those before it, does not represent an endpoint. For example, our study shows that ToL-metabarcoding performs better than ESS by providing more identifiable taxa across the tree of life in the face of an abundance of bacteria. Moreover, the use of multiple assays in ToL-metabarcoding revealed a significant fraction of taxa that would have been missed with a single (universal) assay. That said, despite filtering several one litre replicate samples in this study ($N=9$), the sampling design did not enable a thorough investigation of inter-sample variation. We have therefore yet to explore how repeatable these assays are, or how effective they lend themselves to tracking changes in biodiversity across multiple spatial and temporal scales[25]. Rigorous testing can be achieved by reprocessing all of the samples from the beginning of the workflow to the end and then comparing results, sampling the same location at another time point or additional sampling at more distant sites, but that is beyond the scope of this study. Regardless of the NGS method used, there was always a significant fraction of the sequence data that could not be assigned a taxonomic rank. This deficiency reinforces the need for improved DNA reference databases, but also the awareness that tandemly running taxonomic-independent approaches (i.e. OTU analysis) provides more accurate measures of beta and alpha diversity. Finally, at present, ToL-metabarcoding can be expensive, labour intensive and difficult for more general ecologists to implement when compared to traditional biodiversity survey methods in the field, but this will only continue to decrease with the refinement of metabarcoding lab protocols and availability of commercial services. Moreover, no traditional survey method has yet been able to holistically capture the ecosystem composition across the entire tree of life in the way ToL-metabarcoding can.

Conclusions

The goal of EBM is to consider biodiversity holistically, and in this regard, the taxa identified here using eDNA sourced from seawater is unparalleled in scope by any other survey method yet devised. Further, sampling seawater is easier than other methodologies currently used for assessing biodiversity, as it requires minimal equipment, is rapid, non-invasive, overcomes the need for deploying infrastructure and has the potential to be routinely collected using autonomous gliders or drones[16,18]. With present sequencing technologies and associated costs, we have shown that metabarcoding is superior to ESS in terms of representing ocean biodiversity and for increasing the recovery of non-microbial taxa. Multiple PCR assays should be employed however, that include universal primers, to provide biodiversity snapshots across a broad taxonomic spectrum, and taxon-specific primers, to focus on groups of interest. Once the collection and isolation of DNA from the environment, as well as laboratory and bioinformatics workflows are standardised, the application of eDNA analyses hold great promise for future marine biomonitoring[16,18].

Materials and Methods

Sampling site, water collections and DNA extraction. Nine one litre seawater samples were collected using sterile Nalgene bottles from the surface at Coral Bay, which is located within the World Heritage site of Ningaloo Reef on the northwest coast of Australia. Three samples were collected on the 17th of March 2015 at 9:00 am from Coral Bay jetty (−23.154793, 113.766328), and six samples were collected from within the lagoon (−23.154793, 113.766328); three at 5:30 pm on the 17th of March and three at 6:30 am on the 18th of March. Each one litre seawater sample was filtered across a Millipore 0.2 μm hydrophilic nylon membrane (Merck Millipore, Massachusetts, USA) using a Masterflex peristaltic pump (Cole Palmer, Vernon Hills, USA). The membrane disc containing captured eDNA and cellular material from the water column was immediately frozen and stored at −20 °C. Total nucleic acids were extracted directly from the filter membranes using the DNeasy Blood and Tissue Kit (Qiagen; Venlo, Netherlands) in a dedicated PCR-free DNA extraction laboratory, as well as a blank extraction control, and eluted in 100 μL AE buffer (Qiagen; Venlo, Netherlands).

Shotgun sequencing and analyses. Equal volumes of each of the nine DNA extracts were pooled together and used to prepare a single shotgun library using the Nextera XT DNA library kit (Illumina, San Diego, USA). ESS was performed using a NextSeq® 500/550 v2 high output 300 cycle kit on an Illumina NextSeq system located in the Trace and Environmental DNA (TrEnD) Laboratory at Curtin University. Sequences with an average Q score ≤25 that contained no ambiguous nucleotides and were 151 base pairs (bp) in size (corresponding to the length of a DNA fragment sequenced uni-directionally), were compared to the National Center for Biotechnology Information (NCBI) nucleotide database using BLASTN on the Magnus Cray XC40 system located in the Pawsey Supercomputing Centre at Technology Park in west Australia. Assignment of sequences to taxa at a particular taxonomic level was assessed using the software MEGAN 5.11.3[70] using a Min Score of 100 and a Top Percent of 10 under the LCA Parameters.

PCR metabarcoding and sequencing. PCR was performed in duplicate on each of the nine DNA extracts using ten primer sets containing template-specific oligonucleotides (Supplementary Data 6) that target different taxonomic groups. To reduce the likelihood of cross-contamination, chimera production and index-tag switching[71], amplification of target DNA was performed in a single round of PCR using fusion tag primers consisting of an Illumina adaptor, indexes unique to this study and the template specific oligonucleotide in an ultra-clean laboratory designed for ancient DNA work. PCR reagents included 1 × AmpliTaq Gold® Buffer (Life Technologies, Massachusetts, USA), 2 mM $MgCl_2$, 0.25 μM dNTPs, 10 μg BSA, 5 pmol of each primer, 0.12 × SYBR® Green (Life Technologies), 1 Unit AmpliTaq Gold DNA polymerase (Life Technologies), 2 μl of DNA

and Ultrapure™Distilled Water (Life Technologies) made up to 25 μl total volume. PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Massachusetts, USA) under the following conditions: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 30 s at 95 °C, 30 s at the primer annealing temperature and 45 s at 72 °C, with a final extension for 10 min at 72 °C. Complete primer information and annealing temperatures are provided in Supplementary Data 6. For the three primer sets that target the nuclear 18S and mitochondrial COI genes, PCR was performed using three annealing temperatures in an attempt to maximise template amplification and the diversity of taxa detected. All duplicate PCR products from the same 1 L sample were combined prior to library pooling. PCR negative controls were included for all assays and any taxa detected within them were removed from all samples for analyses.

Libraries for sequencing were made by pooling amplicons into equimolar ratios based on qPCR Ct values and band intensity on a 2% agarose gel stained with ethidium bromide. Amplicons in each library were size-selected using a Pippin Prep (Sage Science, Beverly, USA) and purified using the Qiaquick PCR Purification Kit (Qiagen; Venlo, Netherlands). The volume of purified library added to the sequencing run was determined using qPCR against DNA standards of known molarity as in Murray *et al.*[72]. Depending on the amplicon size, libraries were either unidirectionally sequenced using a 300 cycle MiSeq® V2 Reagent Kit and nano flow cell, or with paired-end sequencing using a 500 cycle MiSeq® V2 Reagent Kit and standard flow cell on an Illumina MiSeq platform located in the TrEnD Laboratory at Curtin University. Sequence data is available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.qq11c.

Metabarcoding analyses. All data generated by Illumina sequencing were filtered through a series of quality control steps prior to taxonomic assignment and OTU analyses. Metabarcoding reads recovered by paired-end sequencing were first stitched together using the Illumina MiSeq analysis software under the default settings. In order to eliminate low quality sequences, only reads matching 100% to Illumina adaptors, index barcodes and template specific oligonucleotides identified using Geneious® 8.1.4.[73] were kept for downstream analyses. For each sample, Mothur 1.36.1[74] was used to remove singletons, sequences that had an average Q score $\leq$25 and reads that contained ambiguous bases. Potential chimeras were identified using Perseus[75] and removed from the dataset. Amplicons originating from eukaryotes that passed quality filtering were queried against the NCBI nucleotide database using BLASTN on the Magnus Cray XC40 system.

Given the lack of reference barcodes for most taxa, which limits the ability to asses inter-species diversity, we used a conservative approach to assign sequences to species, as opposed to percentage sequence similarity thresholds used in other studies[34,52]. Taxonomic identification was assigned to a species only if there was a 100% sequence identity match, if a sequence from at least one other species within the same genus was available for comparison (and $<$100% identical) and if the distribution of the species hit matched online database records for flora and fauna known to the region (e.g. Atlas of Living Australia; http://www.ala.org.au.). Otherwise, the taxonomic resolution achieved for a sequence was collapsed to the genus level or even higher. Taxonomic nomenclature was based on the World Register of Marine Species (WoRMS; http://www.marinespecies.org/). For prokaryotic 16S rDNA sequences, OTUs were identified following the MiSeq SOP outlined in Kozich *et al.*[76] and using the NR SILVA database (release 123) accessed from the Mothur website (http://www.mothur.org/wiki/MiSeq_SOP) on the 2nd of May, 2016. OTU classification for sequences assigned to the fish class Actinopterygii was also parsed using a 98% sequence similarity in Mothur 1.36.1[74]. Rarefaction analyses were performed using Analytic Rarefaction 1.3[77] and plotted using R[78].

Network analyses. To assess haplotype diversity within the commercially targeted fish genus *Lethrinus*, a sequencing error rate for fish 16S rDNA was determined from single source tissue samples and thus used to filter out potential sequence artefacts. To achieve this, DNA extracted from 13 fish species, representing 12 different families (including two *Lethrinus* species), were individually amplified and the resulting 16S rDNA amplicons were sequenced as per above on an Illumina MiSeq. A single dominant haplotype for each species was considered the true representative haplotype for that species, and additional sequences were designated error generated during the PCR process, Illumina cluster formation and/or sequencing. The frequency of the highest erroneous sequence as a percentage of the true haplotype for each species was calculated, and an average error rate for fish 16S rDNA amplicons determined. This error rate was used to filter out low abundance sequences assigned to *Lethrinus* generated by PCR for the eDNA samples collected at Coral Bay when compared to the most abundant sequence assigned to *Lethrinus* in the same sample. In addition, potential *Lethrinus* 16S rDNA haplotypes that surpassed this error threshold needed to be present in multiple PCR libraries amplified from each of the seawater samples collected at Coral Bay. A network of *Lethrinus* 16S rDNA haplotypes recovered from Coral Bay and all available *Lethrinus* 16S sequences on Genbank was constructed in PopART (http://popart.otago.ac.nz). Because indels cannot be treated as a 5th character state in PopART, the alignment was edited to make gaps informative. The Atlas of Living Australia was used to determine the feasibility of *Lethrinus* species that we recorded at Coral Bay.

References

1. Jennings, S. & Polunin, N. V. Impacts of fishing on tropical reef ecosystems. *Ambio* **25**, 44–49 (1996).
2. Pauly, D., Christensen, V., Dalsgaard, J., Froese, R. & Torres, F. Fishing down marine food webs. *Science* **279**, 860–863 (1998).
3. Halpern, B. S. *et al.* A global map of human impact on marine ecosystems. *Science* **319**, 948–952 (2008).
4. Hoegh-Guldberg, O. & Bruno, J. F. The impact of climate change on the world's marine ecosystems. *Science* **328**, 1523–1528 (2010).
5. Worm, B. *et al.* Rebuilding global fisheries. *Science* **325**, 578–585 (2009).
6. Day, J. The need and practice of monitoring, evaluating and adapting marine planning and management – lessons from the Great Barrier Reef. *Mar Pol* **32**, 823–831 (2008).
7. Watson, D. L., Harvey, E. S., Anderson, M. J. & Kendrick, G. A. A comparison of temperate reef fish assemblages recorded by three underwater stereo-video techniques. *Mar Biol* **148**, 415–425 (2005).
8. Richards, Z. T. A comparison of proxy performance in coral biodiversity monitoring. *Coral Reefs* **32**, 287–292 (2013).

9. Palumbi, S. R. *et al*. Managing for ocean biodiversity to sustain marine ecosystem services. *Front Ecol Environ* **7**, 204–211 (2009).
10. Smale, D. A. *et al*. Regional-scale benthic monitoring for ecosystem-based fisheries management (EBFM) using an autonomous underwater vehicle (AUV). *ICES J Mar Sci* **6**, 1108–1118 (2012).
11. Tett, P. *et al*. Framework for understanding marine ecosystem health. *Mar Ecol Prog Ser* **494**, 1–27 (2013).
12. Borja, A. Grand challenges in marine ecosystems ecology. *Front Mar Sci* **1**, 1 (2014).
13. Borja., A. *et al*. Overview of integrative assessment of marine systems: the ecosystem approach in practice. *Front Mar Sci* **3**, 2–20 (2016).
14. Shokralla, S., Spall, J. L., Gibson, J. F. & Hajibabaei, M. Next-generation sequencing technologies for environmental DNA research. *Mol Ecol* **21**, 1794–1805 (2012).
15. Bohmann, K. *et al*. Environmental DNA for wildlife biology and biodiversity monitoring. *Trends Ecol Evol* **29**, 358–367 (2014).
16. Rees, H. C., Maddison, B. C., Middleditch, D. J., Patmore, J. R. M. & Gough, K. C. Review: the detection of aquatic animal species using environmentalDNA – a review of eDNA as a survey tool in ecology. *J App Ecol* **51**, 1450–1459 (2014).
17. Thomsen, P. F. & Willerslev, E. Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. *Biol Conserv* **183**, 4–18 (2015).
18. Barnes, M. A. & Turner, C. R. The ecology of environmental DNA and implications for conservation genetics. *Conserv Genet* **17**, 1–17 (2016).
19. Lydolph, M. C. *et al*. Beringian paleoecology inferred from permafrost-preserved fungal DNA. *Appl Environ Microbiol* **71**, 1012–1017 (2005).
20. Ficetola, G. F., Miaud, C., Pompanon, F. & Taberlet, P. Species detection using environmental DNA from water samples. *Biol Letters* **4**, 423–425 (2008).
21. Taberlet, P., Coissac, E., Hajibabaei, M. & Rieseberg, L. H. Environmental DNA. *Mol Ecol* **21**, 1789–1793 (2012).
22. Foote, A. D. *et al*. Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals. *PLoS One* **7**, e41781 (2012).
23. Pochon, X., Bott, N. J., Smith, K. F. & Wood, S. A. Evaluating detection limits of next-generation sequencing for the surveillance and monitoring of international marine pests. *PLoS One* **8**, e73935 (2013).
24. Willerslev, E. *et al*. Fifty thousand years of Arctic vegetation and megafaunal diet. *Nature* **506**, 47–51 (2014).
25. Bista, I. *et al*. Annual time-series analysis of aqueous eDNA reveals ecologically relevant dynamics of lake ecosystem biodiversity. *Nat Commun* **8**, 1–11 (2016).
26. Sigsgaard, E. E. *et al*. Population characteristics of a large whale shark aggregation inferred from seawater environmentalDNA. *Nat Ecol Evol* **1**, 1–4 (2016).
27. Eisen, J. A. Environmental shotgun sequencing: Its potential and challenges for studying the hidden world of microbes. *PLoS Biol* **5**, 157–162 (2007).
28. Srivathsan, A., Ang, A., Vogler, A. P. & Meier, R. Fecal metagenomics for the simultaneous assessment of diet, parasites, and population genetics of an understudied primate. *Front Zool* **13**, 17 (2016).
29. Venter, C. J. *et al*. Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304**, 66–74 (2004).
30. Danhorn, T., Young, C. R. & DeLong, E. F. Comparison of large-insert, small-insert and pyrosequencing libraries for metagenomic analysis. *ISME J* **6**, 2056–2066 (2012).
31. Sunagawa, S. *et al*. Structure and function of the global ocean microbiome. *Science* **348**, 1261359 (2015).
32. Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C. & Willerslev, E. Towards next-generation biodiversity assessment using DNA metabarcoding. *Mol Ecol* **21**, 2045–2050 (2012).
33. Epp, L. S. *et al*. New environmental metabarcodes for analysing soil DNA: potential for studying past and present ecosystems. *Mol Ecol* **21**, 1821–1833 (2012).
34. Miya, M. *et al*. MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. *R Soc Open Sci* **2**, 150088 (2015).
35. Clarke, L. J., Soubrier, J., Weyrich, L. S. & Cooper, A. Environmental metabarcodes for insects: in silico PCR reveals potential for taxonomic bias. *Mol Ecol Res* **14**, 1160–1170 (2014).
36. Elbrecht, V. & Leese, F. Can DNA-based ecosystem assessments quantify species abundance? Testing primer bias and biomass – sequence relationships with an innovative metabarcoding protocol. *PLoS One* **10**, 1–16 (2015).
37. Zhou, X. *et al*. Ultra-deep sequencing enables high-fidelity recovery of biodiversity for bulk arthropod samples without PCR amplification. *GigaScience* **2**, 4 (2013).
38. Mertes, F. *et al*. Targeted enrichment of genomic DNA regions for next-generation sequencing. *Brief Function Gen* **10**, 374–386 (2011).
39. Dowle., E. J., Pochon, X., Banks, J. C., Shearer, K. & Wood, S. A. Targeted gene enrichment and high-throughput sequencing for environmental biomonitoring: a case study using freshwater macroinvertebrates. *Mol Ecol Res* **16**, 1240–1254 (2016).
40. Valentini, A. *et al*. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Mol Ecol* **25**, 929–942 (2016).
41. Spalding, M. D., Ravilious, C., Grren, E. P. *World atlas of coral reefs*. University of California Press, Berkeley (2001).
42. Parada, A. E., Needham, D. M. & Fuhrman, J. A. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ Microbiol* **18**, 1403–1414 (2016).
43. Marriott, R. J. *et al*. Age-based demographic assessment of fished stocks of *Lethrinus nebulosus* in the Gascoyne Bioregion of Western Australia. *Fisheries Manage Ecol* **18**, 89–103 (2011).
44. Azam, F. & Malfatti, F. Microbial structuring of marine ecosystems. *Nat Rev Microbiol* **5**, 782–791 (2007).
45. Turner, C. R. *et al*. Particle size distribution and optimal capture of aqueous macrobial eDNA. *Meth Ecol Evol* **5**, 676–684 (2014).
46. Hebert, P. D. N. & Gregory, T. R. The promise of DNA barcoding for taxonomy. *Systemat Biol* **54**, 852–859 (2005).
47. Bryant, J. A. *et al*. Wind and sunlight shape microbial diversity in surface waters of the North Pacific Subtropical Gyre. *ISME J* **10**, 1308–1322 (2016).
48. V D Kaars, S. & De Deckker, P. Pollen distribution in marine surface sediments offshore Western Australia. *Rev Palaeobot Palynol* **124**, 113–129 (2003).
49. Morris, R. M. *et al*. SAR11 clade dominates ocean surface bacterioplankton communities. *Nature* **420**, 806–810 (2002).
50. Drummond, A. J. *et al*. Evaluating a multigene environmental DNA approach for biodiversity assessment. *GigaScience* **4**, 46 (2015).
51. Kelly, R. P. *et al*. Genetic and manual survey methods yield different and complementary views of an ecosystem. *Front Mar Sci* **3**, 283 (2017).
52. Leray, M. & Knowlton, N. DNA barcoding and metabarcoding of standardized samples reveal patterns of marine benthic diversity. *Proc Nati Acad Scie USA* **112**, 2076–2081 (2015).
53. Pochon, X., Zaiko, A., Hopkins, G. A., Banks, J. C. & Wood, S. A. Early detection of eukaryotic communities from marine biofilm using high-throughput sequencing: an assessment of different sampling devices. *Biofouling* **31**, 241–251 (2015).
54. Pearman, J. K., Anlauf, H., Irigoien, X. & Carvalho, S. Please mind the gap - Visual census and cryptic biodiversity assessment at central Red Sea coral reefs. *Mar Environ Res* **118**, 20–30 (2016).
55. Thomsen, P. F. *et al*. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. *PLoS One* **7**, e41732 (2012).
56. Olds, B. P. *et al*. Estimating species richness using environmentalDNA. *Ecol Evol* **6**, 4214–4226 (2016).

57. Harvey, E. S., Cappo, M., Butler, J., Hall, N. & Kendrick, G. A. How does the presence of bait as an attractant affect the performance of remote underwater video stations in assessments of demersal fish community structure? *Mar Ecol Prog Ser* **350**, 245–254 (2007).
58. Goatley, C. H. R., González-Cabello, A. & Bellwood, D. R. Reef-scale partitioning of cryptobenthic fish assemblages across the Great Barrier Reef, Australia. *Mar Ecol Prog Ser* **544**, 271–280 (2016).
59. Long, E. O. & Dawid, I. B. Repeated genes in eukaryotes. *Ann Rev Biochem* **49**, 727–764 (1980).
60. Schloss, P. D., Gevers, D. & Westcott, S. L. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. *PloS One* **6**, e27310 (2011).
61. Deagle, B. E., Jarman, S. S., Coissac, E., Pompanon, F. & Taberlet, P. DNA metabarcoding and the cytochrome c oxidase subunit 1 marker: not a perfect match. *Biol Lett* **10**, 20140562 (2014).
62. Ward, R. D., Hanner, R. & Hebert, P. D. N. The campaign to DNA barcode all fish, FISH-BOL. *J Fish Biol* **74**, 329–356 (2009).
63. Stat, M., Carter, D. & Hoegh-Gulberg, O. The evolutionary history of *Symbiodinium* and scleractinian hosts –symbiosis, diversity, and the effect of climate change. *Perspect Plant Ecol Evol* **8**, 23–43 (2006).
64. Stat, M., Yost, D. M. & Gates, R. D. Geographic structure and host specificity shape the community composition of symbiotic dinoflagellates in corals form the Northwestern Hawaiian Islands. *Coral Reefs* **34**, 1075–1086 (2015).
65. Pochon, X. & Gates, R. D. A new *Symbiodinium* clade (Dinophyceae) from soritid foraminifera in Hawai'i. *Mol Phylogenet Evol* **56**, 492–497 (2010).
66. Stat, M., Morris, E. & Gates, R. D. Functional diversity in coral-dinoflagellate symbiosis. *Proc Nat Acad Sci USA* **105**, 9256–9261 (2008).
67. Thomas, L., Kendrick, G., Kennington, W., Richards, Z. & Stat, M. Exploring *Symbiodinium* diversity and host specificity in *Acropora* corals from geographical extremes of Western Australia with 454 amplicon pyrosequencing. *Mol Ecol* **23**, 3113–3126 (2014).
68. Lejzerowicz, F. *et al*. High-throughput sequencing and morphology perform equally well for benthic monitoring of marine ecosystems. *Sci Rep* **5**, 13932 (2015).
69. Simpson, C. J., Cary, J. L. & Masini, R. J. Destruction of corals and other reef animals by coral spawn slicks on Ningaloo Reef, Western Australia. *Coral Reefs* **12**, 185–191 (1993).
70. Huson, D. H., Auch, A. F., Qi, J. & Schuster, S. C. MEGAN analysis of metagenomic data. *Genome Res* **17**, 377–386 (2007).
71. Esling, P., Lejzerowicz, F. & Pawlowski, J. Accurate multiplexing and filtering for high-throughput amplicon-sequencing. *Nucleic Acids Res* **43**, 2513–2524 (2015).
72. Murray, D. C., Coghlan, M. L. & Bunce, M. From benchtop to desktop: Important considerations when designing amplicon sequencing workflows. *PloS One* **10**, e0124671 (2015).
73. Kearse, M. *et al*. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**, 1647–1649 (2012).
74. Schloss, P. D. *et al*. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *App Environ Microbiol* **75**, 7537–41 (2009).
75. Quince, C., Lanzen, A., Davenport, R. J. & Turnbaugh, P. J. Removing noise from pyrosequenced amplicons. *BMC Bioinformatics* **12**, 38 (2011).
76. Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K. & Schloss, P. D. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *App Environ Microbiol* **79**, 5112–5120 (2013).
77. Holland, S. M. Analytic Rarefaction 1.3 starta.uga.edu/software/anRareReadme.html (2003).
78. R Development Core Team. R: *A language and environment for statistical computing*, reference index version 2.2.1. R foundation for statistical computing, Vienna. ISBN 3-900051-07-0. Available from http://www.r-project.org (2008).

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Author Contributions

M.S. and M.B. conceived the ideas and designed methodology; R.B. collected samples; M.S. and T.E.B. designed metabarcoding assays; M.S. performed research; M.S. M.J.H., J.D.D. analysed data, all authors wrote the paper.

Additional Information

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